THE GENUS *LOLIUM*; TAXONOMY AND GENETIC RESOURCES

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The genus *Lolium*; taxonomy and genetic resources.

Proefschrift

ter verkrijging van de graad van doctor in de
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STELLINGEN

1. De meest effectieve manier een genenbank te gebruiken ligt in het zorgvuldig ordenen van de accessies naar herkomst, en daarna het aanhouden van een set verschillende monsters binnen deze goed gedefinieerde herkomsten, en het aan de veredelaar beschikbaar stellen die de set onder lokale omstandigheden kan evalueren.

   J.P. Peeters, H.G. Wilkes en N.W. Galwey. TAG 80: 110-112 (1990).

2. Het herhaaldelijk omroepen van vertragingen van drie kwartier bevordert het klantvriendelijke imago dat de NS beogen niet.

3. De uitgave van de eerste aio-onderwijsgids in het najaar van 1993 toont aan hoe goed voorbereid men aan de invoering van het aio-stelsel begonnen is in 1986.

4. "Een gevolg van data-communicatie is de inflatie van kennis. Nu we alles kunnen opvragen, hoeven we niets meer te weten. Wie dat nog wel wil is de klos: de assistent-in-opleiding (aio) die voor zijn onderzoek elke letter wil lezen. Hij weet wat er te koop is, maar beseft dat hij dit nooit zal kunnen verwerken".

   Prof. E. Andriessen, Volkskrant 5 maart 1994.

5. Het op basis van het weglaten van onderscheidende kenmerken in de verwantschapsanalyse concluderen dat twee taxa nauw verwant zijn, betekend manipulatie van de conclusie.

   n.a.v. Z. Bulinska-Radomska en R.N. Lester. Plant Sys. & Evol. 159: 217-227 (1986).

6. Het verzamelen van vegetatief materiaal van Engels raaigras leidt tot een betere afspiegeling van de populatiestructuur dan het verzamelen van generatief materiaal.

   B.F. Tyler, K. Chorlton en I. Thomas, 1984. IBPGR training courses.

7. Het succes van biochemische markers in biodiversiteitsstudies kan gedeeltelijk voorspeld worden aan de hand van het compatibiliteitssysteem van de te bestuderen soort.

8. Stellingen maken is een mooie traditie, alleen voor wie is nog niet bekend.

Stellingen behorende bij het proefschrift "The genus *Lolium*; taxonomy and genetic resources" Wageningen, 8 april 1994, Birgit P. Loos.

Aan mijn ouders
en Sebastiaan

CONTENTS

GENERAL INTRODUCTION

The grass genus *Lolium* is mainly characterized by the spike. The individual spikelets are placed edgewise, alternating in the concavities of the rachis. All spikelets, except the terminal one, only bear the upper glume (Figure 1). This typical shape of the spike is the reason that the genus *Lolium* is distinguished, and prevents it from being merged in the genus *Festuca*. However some authors (e.g. Stebbins, 1956) argue that the differences of the inflorescence are too small to justify separation of the genera. Some authors present experimental data (e.g. Essad, 1954; Bulinska-Radomska & Lester, 1988) to justify the merging of both genera, but for all practical purposes both taxa are still recognized. This thesis focuses on the intra-generic variation of *Lolium*. Special attention has been given to the taxonomy of the genus and the genetic resources of *Lolium perenne* L.

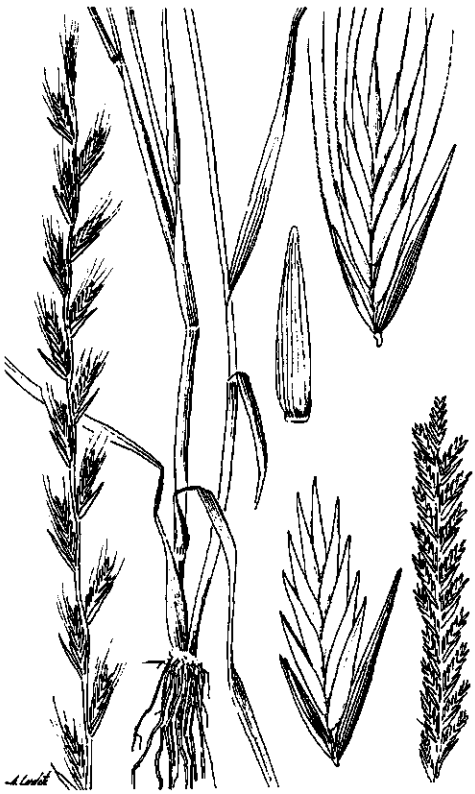

Figure 1: drawing of *L. multiflorum*, typical for the genus *Lolium* (Valdes et al., 1987)

Taxonomic subdivision of the genus *Lolium*

During the past centuries various authors subdivided the genus *Lolium* into several taxonomic groups above the species level, such as subgenera and sections. In 1823 Dumortier published three sections within the genus *Lolium*. Two of these sections were named by the author; section *Ctenium* Dum. and section *Dolathera* Dum. The third section has first been given generic rank by Schrank (1789); sect. *Craepalia* (Schrank) Dum. Section *Ctenium* is described as having unawned flowers, section *Dolathera* has a flexuous rachilla and section *Craepalia* is described as having flowers with straight awns. This does not agree with the description Schrank (1789) gave of the genus *Craepalia*, which differs from *Lolium* as all spikelets bear both glumes. This fact gives rise to the notion that Schrank describes a plant from the genus *Festuca*, and confuses it with a plant known at that time as *Lolium temulentum* L. Godron and Grenier (1848) described two sections within the genus *Lolium*: section *Craepalia* (Schrank) Godr. and

a new section: *Eulolium*. The latter is diagnosed by the elliptic shape of the spikelets, the former has lanceolate spikelets. *L. perenne* L. and *L. multiflorum* Lam. are considered part of the section *Eulolium*, *L. temulentum* belongs to section *Craepalia*. Döll (1857) divided the genus *Lolium* in two subgenera: *Lobeter* and *Dasycholo*, based on the difference in life cycle. Subgenus *Lobeter* contains the annual species, subgenus *Dasycholo* the perennial. Ascherson and Graebner (1902) distinguished four sections: again section *Craepalia* and section *Eulolium*, and two new sections, section *Crypturus* (Link) Asch. & Grab. and section *Monerma* (Beauv.) Asch. & Grab. Section *Crypturus* refers to a generic name introduced by Link (1843). Link mentions *Rottboellia* as a synonym of *Crypturus*. In 1914 *Rottboellia* was put into synonymy of *Lolium loliaceum* by Handel-Mazzetti. The section *Monerma* is diagnosed by the single flower in each spikelet. Terrell (1968) calls this a superfluous name.

In Russian literature the genus *Lolium* is not only divided in sections, but also in series (Nevski, 1934). Two sections are recognized: *Craepalia* and *Eulolium*. Section *Craepalia* contains one series: *Temulentae* Nev., diagnosed as having glumes longer than or equal to the spikelet, 1.2 - 3.0 cm long. Section *Eulolium* contains three series: *Rigidae* Nev., *Multiflorae* Nev. and *Perennes* Nev. This section is described as having thin, herbaceous, lanceolate lemmas. The three series are separated on basis of the length of their life cycle and the presence or absence of awns.

The characters used for the recognition of divisions above the species level within the genus *Lolium*, are seldom clear-cut. Characters as presence of awns, shape of the spikelets etc. also show considerable variation within populations of the *Lolium* species. This makes consistent use of these subdivisions difficult. The relationship between the several species within this genus is so close (Terrell, 1968; Chapters 2, 3 and 4, this thesis) that it seems hardly appropriate to use any of these divisions.

Species within the genus *Lolium*

More than 480 names of various rank have been published during the last two centuries to classify the variation found within the genus *Lolium*. Terrell (1968) has put an enormous amount of work in reordering the species and recognizing synonyms, superfluous names, etc. His revision of the genus *Lolium* is the latest treatment of the genus. Starting point for the description of the species names used in this thesis starts from the classification as given by Terrell (1968). He recognises eight species within the genus *Lolium*: *L. perenne* L., *L. multiflorum* Lam., *L. rigidum* Gaud., *L. remotum* Schrank, *L. temulentum* L., *L. persicum* Boiss. & Hoh., *L. subulatum* Vis. and *L. canariense* Steud. One of these species, *L. rigidum*, is subdivided into two varieties: *L. rigidum* Gaud. var. *rigidum* and *L. rigidum* var *rottbollioides* Heldr. ex Boiss. All these species are treated chronologically, to elucidate their names in this thesis.

L. perenne L. and *L. temulentum* L.

The first species recognized in the genus *Lolium* are *L. perenne* and *L. temulentum*. Both these species are originally described by Linnaeus in the *Species plantarum* (1753, Ed. I). The description of these species by Terrell (1968) is satisfactory. The typification of both

species is reported in Chapter 1, as part of the Linnaean Plant Name Typification Project (Cannon et al., 1983).

L. multiflorum Lam.

In 1778 Lamarck described a third *Lolium* species, *L. multiflorum*. According to Lamarck the main character separating it from the other two species is the higher number of florets within a spikelet. In the description of this new species the robustness of the plants is pointed out, referring to the larger culm length, larger foliage and greater number of spikelets. Also the awns on the florets are mentioned, a remark is made on the fact that the figure from Vaillant (1727) cited as a synonym, pictures a specimen with much more and larger awns than the specimen of Lamarck. This illustrates the fact that already at that time considerable variation existed for a character which is nowadays used as diagnostic for the species. The type specimen (P) is a lectotype, and is illustrative for the higher number of florets and the awns. The other diagnostic characters, such as the robustness of the plants and the larger foliage cannot be seen due to the absence of leaf material on the sheet.

L. remotum Schrank

In 1789, a fourth species has been recognized, *L. remotum* Schrank. The distant location of each spikelet in the spike and the very short seeds are used as diagnostic characters. In the further description of the species only the compact structure of the spikelets and the fact that a spikelet contains 5-8 florets are mentioned. No type specimen is known for this species name.

L. rigidum Gaud., *L. subulatum* Vis. and *L. loliaceum* Hand.-Mazz.

L. rigidum was added to the genus by Gaudin in 1811. Diagnostic characters for this species are the spikelets with only few florets, the long glumes, the long culms, the broad-spreading ears and the annual growth habit. In the extensive description a.o. the red-colouring of the culm, the compression of the spikelets against the spike and the unawned florets are mentioned. The holotype of this species is in the herbarium of the Musée Botanique Cantonal in Lausanne (LAU). Terrell (1968) described two varieties of *L. rigidum* : *L. rigidum* var. *rigidum* and *L. rigidum* var. *rottbollioides* Heldr. ex Boiss. for which *Rottboellia loliacea* Bory & Chaub. is cited as a synonym. A part of *L. loliaceum* Hand.-Mazz. is also referred to as a synonym by Terrell (1968). In the diagnostic key to these two varieties he mentions the thicker rachis, and the shorter culms with, generally, a more prostrate growth habit of *L. rigidum* var. *rottbollioides*.

L. subulatum was first described by Visiani in 1842. Terrell (1968) cites *L. loliaceum* pro parte as synonym. The diagnostic characters given in the Flora Dalmatica (1:90) are the oblong-lanceolate form of the spikelets, the three florets within the spikelets without awns, the glume that presses strongly against the rachis and the annual growth habit. The description of the specimen by Visiani is very extensive and mentions a.o. the length of the spike compared to the total culm length. The figure of *L. subulatum* (Fl. Dal. 1: pl. 3) agrees well with the description. The type sheet of *L. rigidum* var. *rottbollioides* (G), which is also the type for *L. loliaceum* and is originally used as the

type for *Rottboellia loliacea*. Terrell (1968) indicates that on this sheet the specimen on the left agrees with his perception of *L. rigidum* var. *rottbollioides*, but the specimen on the right seems more of an intermediate between this variety and *L. subulatum*. Studying the description Terrell (1968) gave of both taxa and the herbarium specimens listed in his description, no solid criteria on which both taxa can be distinguished are found. Generally *L. rigidum* var. *rottbollioides* is shorter, but occasionally specimens are as tall as *L. subulatum*. Other differences reported are; *L. subulatum* has 1-2 nodes below the spike, *L. rottbollioides* is supposed to have 2-4 nodes. Again this character shows some overlap between the taxa and is difficult to observe. The length of the spike is also mentioned as distinguishing character. *L. rigidum* var. *rottbollioides* should have a spike of about 3 - 11 (-20) cm long, *L. subulatum* has 16 - 25.5 cm long spikes. Again, the studied herbarium specimens (most of them determined by Terrell (1968)) show overlap in this character. A difference in habitat is reported (Terrell, 1968) between *L. subulatum* and *L. rigidum* var. *rottbollioides*. The former is found in fields and waste places in Cyprus, Israel, Lebanon, Syria and former Yugoslavia. The latter is found in the Mediterranean region, the Middle East and north Africa, along roadsides, waste places but also on sandy areas, often in maritime habitats. In conclusion, the delimitation of both taxa is not clear, it can be questioned whether *L. rigidum* var. *rottbollioides* and *L. subulatum* are not merely the smaller and larger version of the same taxon. As the environmental variation within the *Lolium* species is known to be large, one can argue that the found variation is due to differences in growth habitat. Several authors (e.g. Bor, 1968, 1970; Kloot, 1983) treat both taxa as one with the name *L. loliaceum* (Bory & Chaub.) Hand.-Mazz. Von Handel-Mazzetti described this species in 1914, and refers to *Rottboellia loliacea* Bory & Chaub. as basionym and *L. subulatum* as synonym. The holotype is in Geneva (G).

Jenkin (1954) gave a description of *L. loliaceum* as it was used in his crossing experiments. This description agrees well with the present concept of *L. loliaceum*. The most important conclusion that can be drawn from this publication is that *L. loliaceum* is a self-fertilizing species, which makes the status as a species instead of a variety of *L. rigidum* clearer. Specimens examined, determinated by Terrell (1968):

L. rigidum var. rottbollioides Heldr.ex Boiss.

ISRAEL: Judean Mountains, Agua Bella, D. Zohary, 3 May 1951, Fl. Pal. 11954 (HUJ); Mt. Carmel, Shumarigh, Naftolsky, 15 May 1929, Fl. Pal. 11953 (HUJ).

ex-YUGOSLAVIA: in arenosis maritimus ad pagum Fasana, Freyn, 3 June 1877 s.n. (W).

GREECE: Leros, in Pharmacusarum, Heldreich, 6 May 1877 (W); Rhodos, in arenosis maritimus a promont., K.H. & F. Rechinger 8390 (W).

L. subulatum Vis.

ISRAEL: Jerusalem, M. Zohary, 3 March 1924, L48 (HUJ); Ammon, env. of Ein Suella, Eig, Zohary, & Feinbrunn, 8 May 1927, L52 (HUJ); Gile'ad, Wadi Warran, Eig, Zohary, & Feinbrun, 10 May 1927, L50 (HUJ); Gile'ad, Wadi Warran, Eig, Zohary, & Feinbrun, 10 May 1927, L41 (HUJ); Judean Mts., Jerusalem, in wheat fields, Zohary, 2 April 1941, L267 (HUJ); Ammon env. of Ein Suella, Eig, Zohary & Feinbrun, 8 May 1927, L42 (HUJ).

LEBANON: In arenosis firope flun. Beyrouth, Peyron, 17 May 1887, Fl. Syrica N1183 (G); Libano: Aley, Peyron, 17 July 1888, Fl.

Syrica N1183 (G).

L. persicum Boiss. & Hoh.

L. persicum was first described by Boissier & Hohenacker (1853). It is described as erect growing, having 5-7 spikelets scattered on the rachis, awned florets and a glume equal or almost equal to the spikelet. The isotype specimens (G) are very immature, which has probably led to the somewhat small dimensions used to originally describe the species. Terrell (1968) also describes mature specimens.

L. canariense Steud.

The last species listed by Terrell (1968) is *L. canariense* Steud. Steudel (1854) described the species as an annual awned species with glumes somewhat shorter than the spikelet and with a very restricted distribution area: the Canary Islands. Terrell (1968) added to this rather poor description the facts that the florets are long and narrow, the great length of the awns and the glume. He cited two names as synonyms: *L. gracile* Parl. and *L. inflex* Rouv. The lectotype specimen (P) agrees well with the description, but it is also very similar to the immature specimens used as isotypes for *L. persicum* (G). The specimen used as lectotype for *L. gracile* (FL) cited as synonym for *L. canariense*, also looks like an immature *L. persicum* plant. Terrell (1968) mentioned that the status of *L. canariense* can be doubted, he stated that it has most affinities with some *L. multiflorum* types. At present it appears that the affinities are closest to *L. persicum*. The breeding behaviour of *L. canariense*, presently unknown, can give an indication whether *L. canariense* has most affinity with either *L. multiflorum* or *L. persicum*, as the former is a cross-breeding species and the latter an inbreeding species.

In general, Terrell's (1968) classification of the genus *Lolium* is followed, with few exceptions. There are three crossbreeding species: *L. perenne*, *L. multiflorum* and *L. rigidum*. Description and typification of these species are according to Terrell (1968). There are four inbreeding species: *L. remotum*, *L. temulentum*, *L. persicum* and *L. loliaceum*. For *L. remotum* and *L. persicum* the description and, if possible, the typification of Terrell (1968) is followed. The description of *L. temulentum* is according to Terrell (1968), but the typification is changed (see Chapter 1). *L. loliaceum* is composed of the two taxa described by Terrell (1968) as *L. rigidum* var. *rottbollioides* and *L. subulatum*. This species is based on *Rottboellia loliacea*, holotype in (G). The status of the *L. canariense* is not known, further research should clarify whether this taxon should be given a separate status, or if it should be included in another *Lolium* species. As no seeds of *L. canariense* were available during earlier parts of this study, no decisions on the status of this species can be made here. Only in the studies, reported in Chapter 4, using seed morphology and seed protein data, one population of *L. canariense* was available. These results seem to indicate that *L. canariense* is a separate species.

To illustrate and define the variation within the several *Lolium* species, herbarium specimens in the British Museum (BM) and in Kew herbarium (K) were studied as reference material.

L. perenne L.

BULGARIA: In graminosis ad Sophia. 12 July 1922, V. Grigorieff s.n. (BM).

CRETE: Distr. Hierapetra: Montes Aphendi kavusi; inter vineas ad Thriphti, substr. schist. ca 800 m. K.H. Rechinger 13258 (BM).

DENMARK: On dry slope. East of Madum Sö at Rold forest. 15 July 1969, S. Jeppesen & P. Pedersen s.n. (BM).

EGYPT: Baharia oasis, garden of Iran company resthouse. 20 January 1979, M.M.A. Elghaim s.n. (K).

GERMANY: Kappeln, Lehnwiesen. 29 Juni 1886, E. Fuchs s.n. (BM).

HUNGARY: Comit. Pest. ad vivas prope pagnum Szent-Ivan, alt. 190 m. 25 May 1900, A. de Degen & C. de Flatt 288 (BM).

ITALY: Tirol austral. in loc. incultis arenosis pr. Riva ad Benacum. 30 May 1870, leg. rev. Porta s.n. (K).

POLAND: Polonia meridionalis ditio Cracoviae: suburbium Bielany. In confino silvae et agri, ad semitan. 28 June 1973, T. Tacik & J. Neçka 520 (BM).

ROUMANIA: In pascius siccus, ad pag. Corbeanca, alt. 80 m. 2 July 1956, S. Dinulescu & El. Dobrescu s.n. (BM).

SARDINIA: arrondissement de Tempio. 3 July 1882, Asfossado, marais 271 (BM).

SWEDEN: Skåne: Limnhamn. 22 June 1900, Th Nilsson s.n. (BM).

L. multiflorum Lam.

BRITISH ISLES: Oxfordshire, Wolvercote Oxford; on publish trip. 16 July 1945, B.E. Hubbard 12853 (K).

DENMARK: Jutland, Bjöstrup at Kalö. 8 August 1969, S. Jeppesen 579 (BM).

EGYPT: El Atf. district El Rahmaiya. March 1923, Ahmed Juref Cff. 2149 (K).

FRANCE: Maine-et-Loire, pres d'Angers. 1 June 1868, Heeren s.n. (BM).

GERMANY: Auf Kleeäckern bei Ettlingen in Baden und auf Schüttplätzen bei Karlsruhe in Baden. 2 July 1906, A. Kneucker 716 (K).

GREECE: near the edge of lake Kastoria. 24 May 1983, M. Damanakis 1168 (K).

HUNGARY: Comit. Pest. secus viam ferream prope Aquincum, alt. 110 m. 1 July 1900, C. de Flatt 290 (BM).

NETHERLANDS: Cultivated fields on löss soil, above Gulpen (prov. Limburg) subspontaneous. 4 June 1952, K.U. Kramer & E.A. Mennega,s.n. (K).

SICILY: Caltanisetta c 20 km S of Piazza Armeria, alt. 100 m. 17 May 1979, D. Davis & S. Sutton 63268 (BM).

SWEDEN: Skåne, Malmo. August 1876, B. Jonsson & E.W. Cedervall s.n. (K).

L. rigidum Gaud.

BRITISH ISLES: Newport, Isle of Wright: waste ground. 9 July 1928, R. Snelville s.n. (K).

FRANCE: la Echaubrugue, Vendée. 30 June 1869, G. Genevier s.n. (BM).

IRAQ: 2 km N of Saediya, alt. 105 m. 9 April 1976, Al-Kaisi 44226 (K).

JORDAN: east Jordan. 29 April 1963, J.B. Gillet 15944 (K).

MALTA: vicinity of St. Paulus bay. May 1972, J. Silverwood s.n. (K).

PORTUGAL: coast near Estoril. 5 June 1933, S.C. Atchley s.n. (K).

SICILY: Siracusa; 6 km SE of Pachino Capo Pessaro, sea level. 13 May 1979, D. Davis & S. Sutton D63013 (BM).

SPAIN: dry fields of Cartagena. 26 April 1926, E. Ellman & N.Y. Sandwith 414 (K).

SWITZERLAND: Cieta inter efetes ampeluta ad dabyrintum, in campus Coltae per Gortynae ruinas. May 1846, Heldreich s.n. (K).

TURKEY: in patches of barley among the ruins of Elaeussa Sebaste, 40 km W of Mersim. 22 April 1964, C.C. Townsend 640422/24 (K).

L. loliaceum Hand.-Mazz.

AFGHANISTAN: Obey springs, 100 km E of Herat in mountain valley near water, alt. 2100 m. 13 July 1969 386 (K).

BRITISH ISLES: 12 North Hants, Blackmore: introduced with wool "shoddy". 22 May 1961, M. McCallum 5418 (K).

CRETE: Creta dist. sitia Guduras, in lapidosis ad sinum Mawrijalos. K.H. Rechinger 12815 (K).

CYPRUS: near Dhiorios, 900 ft. by cornfield. 25 March 1962, R.D. Meikle 2338 (K).

EGYPT: Burg al Arab near El-Iskandariya (Alexandria), cultivated fields and orchards on sandy soil near sea level. 20 March 1990, T.A. Cope, A.G. Fahmy & I.A. El-Garf 260 (K).

GREECE: Attica pr. Porto Raphti. May 1929, F.

Guiol 605 (BM).

HUNGARY: Croatia litoralis. In litoralibus prope pagnum Martinscica sol aren alt. 1-2 m. 7 June 1903, T. Vadocz & A. Smoquina 291 (BM).

SLOVENIA: in arenosis maritimus ad pagnum Fasana, Istriae aust. Solo calc. alt 2 m. 3 Juno 1877, J. Freyn s.n. (K).

L. temulentum L.

AFGHANISTAN: Prov. Takhar: Badam-darrah, südlich von Taluqan, 1000 m. 19 June 1965, D. Podlech det. N.L. Bor 11419 (K).

BELGIUM: Moissons à Visé (Liège). 13 July 1869, E. Marchal s.n. (BM).

BULGARIA: Bulgaria occidentalis: in cultis prope pag. Bunovo dist. Sophia. 6 July 1965, B. Kuzmanov & D. Peev s.n. (BM).

CYPRUS: Kambyli, cornfield weed. 30 April 1957, L.F.H. Merton 2989 (K).

FRANCE: dans les champs a Pontailles sur Saône, Cote d'Or. 20 July 1873, Dr. Bonnet s.n. (K).

GERMANY: Osnabrück, auf Schutt am Hafen. 10 October 1931, Dr Preufs s.n. (K).

LUXEMBOURG: plateau a l'est de Bofferange, champ d'Avena sativa sur Grès de Luxembourg. 14 August 1956, L. Reichling s.n. (K).

PORTUGAL: Beira: near Coimbra south of Rio Mondego. 28 May 1936, A.W. Maxwell 1031 (BM).

SPAIN: prepirineos aragoneses, between Sabiñanigo and Fiscal: Basarán, in arable land on slopes above village. 9 July 1956, N.Y. Sandwith 4673 (K).

SWEDEN: in agris ad Kalmar, Smalandie frequenter. July 1868, F. Ahlberg s.n. (BM).

SWITZERLAND: Chaumont, sur Neuchatel. 24 September 1871, Sire s.n. (K).

TURKEY: Manisa: 12 km south of Dermirei, alt. 600 m, roadside. M.J.E. Coode & B.M. Jones s.n. (K).

L. remotum Schrank

BELGIUM: Champs de lin a St Troud. July 1867, H. Vandenborn s.n. (K).

BRITISH ISLES: Co. Donegal Letterkenny in flax field. 4 August 1929, Mrs Wedgewood s.n. (K).

ESTONIA: Near Tartu, Raadi, as weed in a flax field. 27 August 1924, E. Lepik s.n. (K).

FINLAND: Isthmus karelicus par Sakkola, in agro lino consito prope templum. 1 August 1897, H. Lindberg s.n. (K).

HUNGARY: in agris Lini usitatissimi circa Jarvorow, solo arenoso, 235 m. Woloszczak s.n. (BM).

SPAIN: Castilla: Bujedo champs de lin. 16 June 1907, H. Elías 4694 (BM).

SWEDEN: Västergötland, viske klefva. July 1911, R. Vallquist s.n. (BM).

SWITZERLAND: près Perney Vaud Suisse, dans les lins, vers 800 m. August 1880, Past. Cruchet s.n. (BM).

L. persicum Boiss. & Hohen.

AFGHANISTAN: SE Afghanistan, Kandahar: in arenosis deserti registan prope Bhagat, 600 m. 1968, 30°32'N 63°52'E, N.L. Bor s.n. (K).

CANADA: from quite bad infestation, believed to have been introduced in 1943, Northmark, Alta. 22 July 1943, G.W. Shewchuk s.n. (K).

CHINA: Tsinghai: Hsining, shady roadside under trees. 3 August 1944, J.L. Keng, s.n. (K).

IRAN: Khamseh; Qazvin to Hamadan, 4000 ft. alt.; at edge of cultivated land. 27 June 1960, Furse & Synge 684 (K).

TURKEY: Prov. Maraş dist. Goksun: Hobek dağ, 1700 m. Cornfield weed. 21 July 1952, Davis, Dodds & Cetik 20197 (K).

L. canariense Steud.

CANARY ISLANDS: Pl. canariense (1855) ex itenere secundo no 1565, Teneriffa: in udis convallium opacarum. 24 April 1855, Guimar (K); Gomera, San Sebastian, damp spots in lateral barranco off Barranco de la villa ca. 6 km from town. 19 April 1977, S.A. Renvoize 2795 (K); Hierro, forest above Frontera, track through Erica arborea forest. 21 April 1977, S.A. Renvoize s.n. (K).

Genetic variation in *Lolium* species

The classification of the species within the genus *Lolium* has undergone many changes during the last centuries. It is obvious that species limitations are often blurred, which is reflected in the complex classification systems used. Two important factors cause this. First the adaptability of *Lolium* species to the environment. This adaptability causes the great variation of forms known within almost each *Lolium* species, and which often have been identified as new taxa in the past. The second cause is the fact that the cross-breeding species can interbreed, which can lead to the existence of hybrid forms in nature. This implies that the taxonomic/morphologic species concept is used in this thesis and not the biological species concept.

In a morphological study the populations of the several *Lolium* species, except *L. canariense*, are compared in a field trial under the same environmental conditions. This was done to determine the amount of variation found within each species under the same environmental conditions, to determine the relationships between the several *Lolium* species and to evaluate some diagnostic characters. In Chapter 2 the results from this study are reported, and compared with experimental results from literature. Next to the morphological variation, a study on the allozyme variation in the several *Lolium* species is performed. A selection from the populations used in the field trial is analyzed to enhance the comparability of both studies. In Chapter 3 the results from this study are described and compared with the results from Chapter 2. Next to the differences in morphology of the whole plant, Terrell (1968) also uses seed characteristics as diagnostic characters for some *Lolium* species. In Chapter 4 the results are described of the comparison of the seed morphology of the same populations from several *Lolium* species used in Chapter 2. This was done to gain insight in the diagnostic value of these characters, and to look at the intraspecific variation for these characters. Also, seed protein patterns are compared, and the diagnostic value of these patterns is determined.

L. perenne: a variable fodder crop

From an economical point of view *L. perenne* is the most important species within the genus *Lolium*. It has been cultivated for such a long time by man that its centre of origin is not known. According to Terrell (1968) it is indigenous in parts of Europe, Asia and north Africa. But its original distribution area covers the whole of Europe, temperate Asia and north Africa. It has been introduced to almost all the rest of the world. Europe is generally considered as a centre of variation for this species.

There are reports (Beddows, 1953) that in 1677 seeds of *L. perenne* were collected and sold in England. By 1855 Lawson lists ten different populations of *L. perenne* for which seeds can be supplied to sow and improve grassland. Differences in winter hardiness, habitus, spring growth, abundance of foliage, broadness of the leaves and leaf colour are used to describe these ten populations which carry names such as 'Evergreen', 'Spreading', 'Stickney's' and 'Pacey's'. Even advice for which purposes the populations seem most suitable is given, making it look quite like a cultivar list as used in present times. Breeding activities within this species have been for

some time, and still are, very extensive. As breeding largely depends on the genetic diversity available, it is of interest to analyze this diversity. Developments in the last century gave rise to the idea that also for this widespread species genetic diversity is reducing. One of the postulated causes is the very intensive practice of sowing and resowing of grasslands with cultivars. An other cause is the drastic reduction of non-cultivated land in the last decades. In this study the attention has been focused on the genetic diversity for *L. perenne* in the Netherlands. The both causes mentioned are certainly valid for the Netherlands, as there is hardly a country where agricultural practice is so intensive, and where, due to a.o. urbanisation hardly any space is left for the existence of natural populations. Raygrass covers no less than 1.3 million ha of production grassland, that is the largest area of any crop in the Netherlands.

Genetic resources conservation mainly focuses on the most efficient way to preserve genetic diversity. This means optimizing the amount of genetic diversity in a population sample that should be as small as possible. This leads to the formulation of three research questions:

1) do Dutch *L. perenne* populations constitute an essential element of the genetic diversity already sampled in the rest of Europe ?
2) is genetic diversity in Dutch *L. perenne* populations comparable with the diversity found in some much used cultivars ?
3) is *in situ* conservation suitable for the conservation of genetic diversity within *L. perenne* ?

To answer these three questions a collection of Dutch *L. perenne* populations is compared with a number of non-Dutch populations and a selected number of cultivars. Results from the morphological comparison of these objects are reported in Chapter 5. The comparison of the allozyme variation for all these populations is presented in Chapter 6, and results from both chapters are compared. In the general discussion all results are combined to discuss the amount of genetic diversity and the treatment of this genetic diversity within the genus *Lolium*, and more particular for *L. perenne*.

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CHAPTER 1:

THE TYPIFICATION OF *LOLIUM PERENNE* L. AND *LOLIUM TEMULENTUM* L. (*POACEAE*)

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Summary

The typification of the Linnaean species *Lolium perenne* and *Lolium temulentum* has been studied. *Lolium perenne* is typified by material in LINN, as proposed by Terrell, but it has been necessary to select a lectotype for *L. temulentum*, and material in the Burser herbarium (UPS) has been chosen for this purpose. The study shows that although Linnaeus used awns as a diagnostic character to distinguish the two species, he was aware of the intraspecific variability in this character.

Introduction

A project, studying variation in natural populations of *Lolium* in relation to variation in cultivated plants of the same genus is in progress. The Centre for Genetic Resources, the Netherlands (CGN), the Centre for Plant breeding and Reproduction research (CPRO-DLO) and the Department of Plant Taxonomy of the Agricultural University in Wageningen are taking part in this project. Part of this work involves a study of species delimitation in the genus *Lolium* because in agricultural practice today the distinctions between species are blurred. Characters such as the presence of awns which were originally used for species identification are no longer restricted to the species for which they were supposed to be diagnostic. This problem necessitates an unambiguous typification of the *Lolium* species, and accordingly, a study of the typification of *Lolium perenne* L. and *Lolium temulentum* L. has been made in collaboration with the Linnaean Plant Name Typification Project (Cannon, Jarvis & Robson, 1983; Jarvis, 1986).

In the first edition of his *Species Plantarum*, Linnaeus (1753) described two species in the genus *Lolium*, *L. perenne* and *L. temulentum*. *Lolium temulentum* is known under the common name of Darnel and used to be a widespread weed of cereals. Darnel has been known to man for a very long time, Dioscorides having described its medicinal use in the first century A.D. (Gunther, Goodyer & Dioscorides, 1934: 133). *Lolium perenne* is known as perennial ryegrass, and has probably been used as a fodder crop for quite some time. Early botanists such as Ray (1724) had mentioned its feed value for cattle.

Elements of *L. perenne*

The first valid publication of the name *L. perenne* was in the *Species Plantarum* ed. I (1753: 83; Text 1)

Linnaeus provided a new diagnostic phrase-name which characterized the species on the absence of awns. He also cited three synonyms. The first was from his own *Hortus Cliffortianus* (Linnaeus 1738), also cited via

Text 1: Protologue Species Plantarum ed I (1753) p 83, *L. perenne*

1. LOLIUM ſpica mutica.
Lolium ſpicis muticis, radice perenni. *Hort. cliff.* 24[2] *perenne.*
Fl. ſuec. 104. *Roy. lugdb.* 69.
Lolium ſpicis compreſſis, radice perenni. *Fl. lapp.* 32.
Gramen loliaceum, anguſtiore folio & ſpica. *Bauh. pin.*
9. *theatr.* 127. *Scheuch. gram.* 25.
Habitat in Europa *ad agrorum verſuras ſolo fertili.* ⊙

his *Flora Suecica* (1745) and the *Prodromus* of Adriaan van Royen (1740). The second synonym was from Linnaeus' *Flora Lapponica* (1737), and the third from Bauhin (1671), also cited via Bauhin (1658) and Scheuchzer (1719). The annual sign (⊙) was inserted in the text due to an error and was corrected to a perennial sign (♃) in the Appendix of the work.

In the Linnaean Herbarium (LINN, see Savage 1945), there are four sheets bearing material that has been identified as *L. perenne*. Sheet 99.3 is from the Middle East and appears to be a post-1753 addition to the herbarium, whereas sheet 99.4 is not annotated by Linnaeus at all. We do not regard either of these as original material. However, sheets 99.1 (Fig. 1) and 99.8 both bear European material which appears to have been in Linnaeus' possession in 1753 and which is original material. The Linnaean Herbarium in Stockholm (S) contains two sheets bearing material referred to this taxon, but neither is annotated by Linnaeus and we do not regard them as bearing original material.

Turning to the synonyms, there is no relevant material in the Clifford Herbarium (BM), but there is a good specimen (Fig. 2) in the van Royen herbarium (sheet no. 912.356-230, L). Linnaeus worked closely with Adriaan van Royen during the winter of 1737-1738 on a new system for arranging the plants in the Leiden Botanic Garden (Veendorp & Baas-Becking 1938). We regard this sheet as original material. There is no relevant material extant associated with Linnaeus' second synonym, from his *Flora Lapponica* (1737) entry for this taxon. The third synonym, from Bauhin (1671), can be associated with material (vol. I: 115) in the Burser Herbarium (UPS-BURS). This herbarium was arranged according to Bauhins' *Pinax* and as the specimens were in Uppsala throughout Linnaeus' life they served as a voucher collection for Bauhin's names. Linnaeus also provided determinations for most of these specimens (see Stearn 1957). The material of I: 115 has been identified by Linnaeus (in ms., see Savage 1937) as belonging to a species of *Bromus*; the material on sheet I: 114 is rather small, bears small awns and has been determinated by him as *L. perenne*, but the Bauhin polynomial on this sheet is not cited in

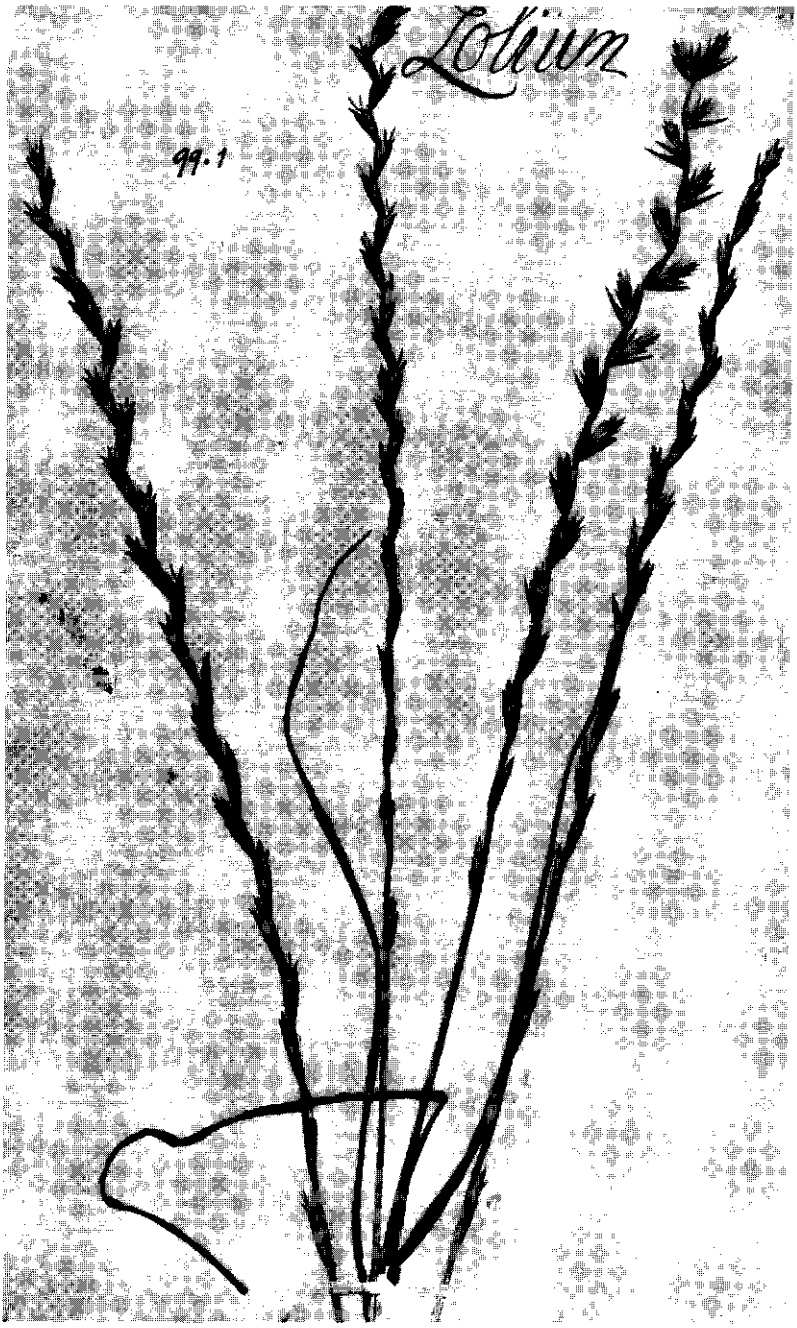

Figure 1: Specimen LINN 99.1: *Lolium perenne*. The lectotype of *L. perenne* designated by Terrell (1968). (Photo by BM)

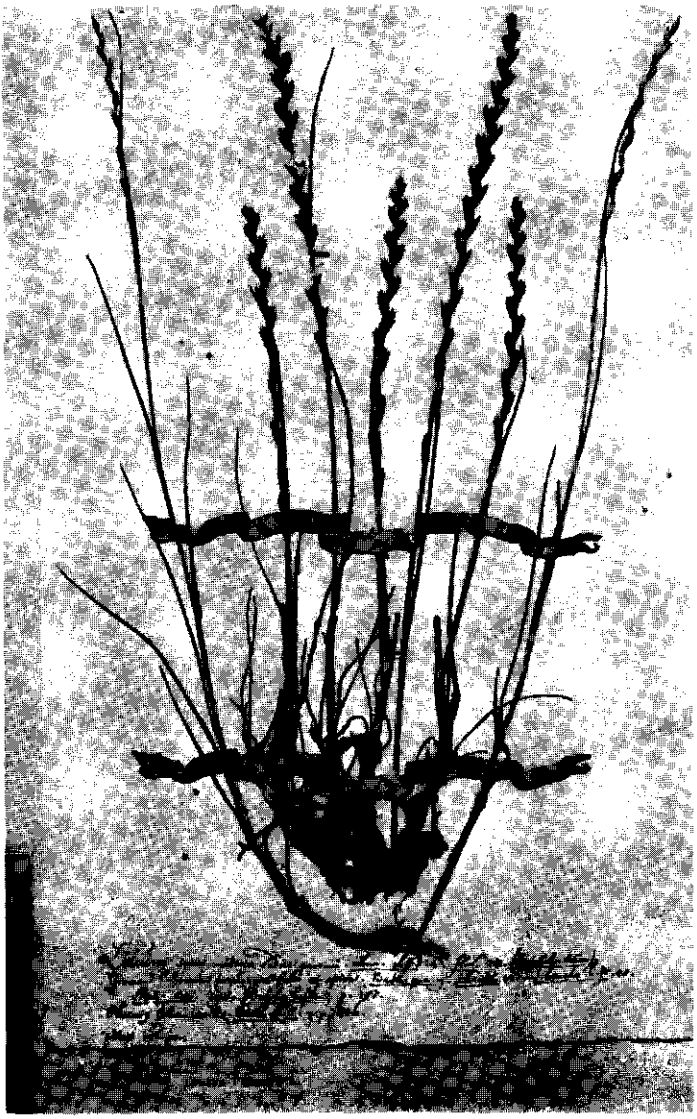

Figure 2: Specimen no. 912.356-230 Adriaan van Royen herbarium (L). *Lolium*. "Lolium spicus muticus, radice perenni".

the protologue of *L. perenne*. In addition, being awned, the material conflicts with Linnaeus' diagnosis.

More extensive descriptions of Bauhin's plant can be found in the accounts cited by Bauhin (1658), where there is an illustration (Fig. 3) depicting a plant with large glumes dissimilar to sheet 115 in UPS, and Scheuchzer (1719).

It is noteworthy that although absent from his 1753 treatment, Linnaeus earlier (1738, 1745) cited an illustration from Morison (1699) in the synonymy of this species. This plate (Fig. 4) is a good picture of the species and the abnormalities found within it. However, it does not constitute a type element.

In his revision of Lolium, Terrell (1968) considered the typification of *L. perenne* and selected the material on sheet 99.1 (LINN) as the lectotype. He noted that it was not typical, but we agree with him that it constitutes "original material". Accordingly, his choice of type must stand as it appears to be the earliest such designation. It has been treated as such by Mill in Davis (1985), and Sherif & Siddiqi (1988: 20) also indicate this collection as the lectotype.

Figure 3: Plate " Gramen loliaceum angustiore folio et spica seu Phoenix dioscoridis" (Bauhin, 1658: 128). (Photo by AU in Wageningen.)

Figure 4: Plate "Gramen loliaceum spica simplici, vulgare" (Morison, 1699: 181). (Photo by BM)

Elements of *L. temulentum*

The first valid publication of the name was in the *Species Plantarum* ed. 1 (1753: 83; Text 2).

Linnaeus provided a new phrase-name (though clearly derived from his earlier *Hort. Cliff.* account), and cited two polynomials in synonymy. The perennial sign (♃) was evidently inserted in the text due to an error and was corrected to an annual (☉) sign in the Appendix of the work.

In the Linnaean herbarium (LINN), there are five sheets referred to *L. temulentum*. Sheet 99.2 was received by Linnaeus in 1756, 99.9 also appears to be a later addition to the herbarium and 99.10 has been referred to this

Text 2: Protologue Species plantarum ed I (1753) p 83.

2. LOLIUM ſpica ariſtata.
Lolium ſpicis ariſtatis, radice annua. *Hort. cliff.* 23. *Fl. temulentum ſuec.* 103. *Roy lugdb.* 69.
Gramen loliaceum, ſpica longiore, ſ. Lolium dioſcoridis.
Bauh. pin. 9. *theatr.* 121. *Scheuch. gram.* 31.
Habitat in Europæ *agris inter Hordeum, Linum.* ☿

taxon only by J. E. Smith. None of these collections is regarded here as original material. Sheets 99.5 and 99.7, although apparently in Linnaeus' possession by 1753 and regarded by him as belonging to this taxon, both bear material which is unawned, and which therefore conflicts with his own diagnosis. Neither of these collections can be regarded as potential type material. There is also a sheet referred to the species in the Linnaean Herbarium in Stockholm (fiche 45.19), but it is not annotated by Linnaeus and is not original material.

Linnaeus' first synonym is from his *Hortus Cliffortianus* (1738), also cited via his *Flora Suecica* (1745) and van Royen (1740). Although there is a sheet in the Clifford herbarium (BM) referred to this name, the material is unawned and therefore conflicts with Linnaeus' diagnosis (Linnaeus 1738, 1753). Consequently it cannot be original material for this name. No relevant material in the van Royen herbarium has been traced.

Linnaeus' second synonym is from Bauhin (1671), also cited via Bauhin (1658) and Scheuchzer (1719). There is material associated with Bauhin's polynomial in the Burser herbarium (vol. I: 113, UPS) which Linnaeus has also explicitly identified with *L. temulentum* (Savage 1937). The material (Fig. 5) is in good condition and belongs to *L. temulentum* as currently understood (see Juel 1936), and is original material. Bauhin (1658) figures an unawned form of *L. temulentum* (Fig. 6), and further detailed descriptions are provided by Scheuchzer (1719)

Previous to the first edition of the *Species Plantarum*, Linnaeus (1738, 1745) cited an illustration "*Lolium verum*" from Morison (1699) in the synonymy of this species (Fig. 7). This plate shows awned and unawned forms of *L. temulentum*, but does not constitute an original element.

Terrell (1968: 35) designated sheet 99.10 LINN as the lectotype of *L. temulentum*, but this collection does not form part of the original material for the name. The only annotations by Linnaeus on the sheet are the words "altitudo humana" and "an *L. perenne*" on the verso of the sheet. The only reference to "temulentum" is written on the front of the sheet in the form of "temulentum Mss" by J. E. Smith after his acquisition of the Linnaean

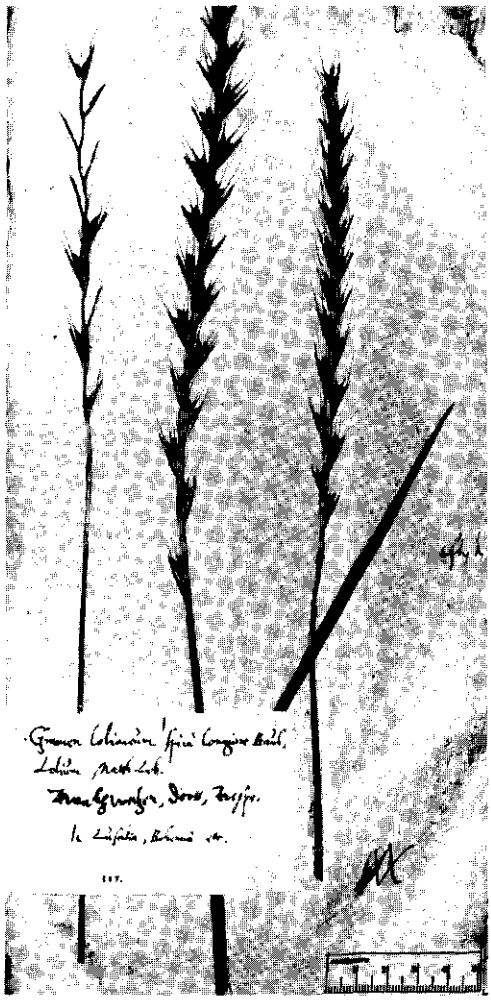

Figure 5: Vol I, folio 113 in the Burser herbarium (UPS-BURS) named "Gramen loliaceum spica longiore" (photo Uppsala herbarium). Lectotype of *L. temulentum* designated here.

herbarium in 1784. There is no evidence that Linnaeus considered this collection to belong to *L. temulentum* (indeed it appears that he thought it closer to *L. perenne*), and as it also seems extremely likely to have been added to the herbarium after 1753, it cannot be regarded as original material and is hence ineligible for selection as a lectotype. Hubbard (1970: 41) indicated unspecified material in LINN, but as a number of collections exists, it is not clear which one he intended to indicate.

A lectotype is therefore required for *L. temulentum*. Neither the material in the

Figure 6: Plate "Gramen loliaceum spica longiore seu lolium dioscoridis" (Bauhin, 1658: 121). (Photo by AU in Wageningen)

Figure 7: Plate *Lolium verum* Gesner (Morison, 1699: 181). (Photo by BM)

Clifford herbarium (BM) nor that in LINN is eligible, as explained above, and this leaves us with a choice between the Burser material and the Bauhin illustration. As the Burser material is in good condition, we prefer it to an illustration, and we here formally designate the material (central inflorescence) in Herb. Burser, vol. I: 113 (UPS) as the lectotype of *Lolium temulentum*.

Conclusions

Lolium perenne L. *Sp. Pl.* 1: 83 (1753). LT.: 99.1 (LINN), designated by Terrell, USDA Bull. no. 1392: 7 (1968).

Lolium temulentum L. *Sp. Pl.* 1: 83 (1753). LT.: Herb. Burser, Vol. I, fol. 113 (UPS-BURS), designated here.

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CHAPTER 2:

MORPHOLOGICAL VARIATION IN *LOLIUM* (*POACEAE*) AS A MEASURE OF SPECIES RELATIONSHIPS

B. P. Loos

Plant Systematics and Evolution (1993) : in press

Summary

Analysis of morphological and phenological data for determining the genetic variation within seven *Lolium* species led to the recognition of two groups within this genus. One group, containing the two inbreeding species *L. temulentum* and *L. persicum*, was clearly distinct from all other species. Strong morphological and phenological intergradation was found between both species. The cross-breeding species, *L. perenne*, *L. rigidum* and *L. multiflorum*, formed another group. Little differentiation was found between these species, though they were distinct. Two inbreeding species, *L. loliaceum* and *L. remotum*, were clearly distinct from each other and the two groups. *L. loliaceum* had an isolated position and was most related to *L. rigidum*. *L. remotum* had an intermediate position between the cross-breeding and inbreeding species, and was almost equally distant from all three cross-breeding species.

Introduction

The genus *Lolium* L. (*Poaceae*) is native to Europe, temperate Asia and North Africa (Terrell, 1968), but has been introduced over almost all of the rest of the world. In general, seven species are distinguished: *Lolium perenne* L. (perennial ryegrass), *L. multiflorum* Lam. (Italian ryegrass), *L. rigidum* Gaud., *L. loliaceum* Hand.-Maz., *L. persicum* Boiss.&Hohen. ex Boiss., *L. temulentum* L. and *L. remotum* Schrank. The first three species are wind-pollinated, cross-breeding species, the latter four are selfing species. All species are diploids (2n=14), but due to breeding activities tetraploid cultivars of the fodder crop species, perennial and Italian ryegrass, are available. Reports on natural hybrids between the cross-breeding species are numerous. Crossing experiments have shown (Jenkin, 1954a,b,c,d) that these crosses are easily made and result in good seed set and germination, and high fertility of the F_1 pollen. Natural hybrids of the selfing species do not occur, although crossing experiments (Jenkin, 1954e) showed that crossing is possible. Natural hybrids between cross-breeding and selfing species do not occur, but crossing was established by Jenkin (1954a,b,c,d).

Species distinction within the genus *Lolium* has been investigated by many authors, using a variety of techniques: analysis of morphological data (Essad, 1954; Naylor, 1960; Terrell, 1968; Kloot, 1983; Bulinska-Radomska & Lester, 1985,1988), analysis of crosses (Jenkin, 1954a,b,c,d,e), nuclear characteristics of crosses (Naylor, 1960; Rees & Jones, 1967), karyotypic studies (Essad, 1954), DNA-content determinations (Malik &

Thomas, 1966), Giemsa banding patterns of chromosomes (Thomas, 1981) and biochemical characteristics (Bulinska-Radomska & Lester, 1985). In general, the genus *Lolium* can be divided into two groups based on breeding system. Within the inbreeding group, species can be separated on the basis of morphological differences (Kloot, 1983), biochemical characteristics (Bulinska-Radomska & Lester, 1985), and from results of crosses (Rees & Jones, 1967). Within the cross-breeding group, species distinction is more difficult. Morphological (e.g. Vasek & Ferguson, 1963; Terrell, 1968; Kloot, 1983; Bulinska-Radomska & Lester, 1985) and biochemical (Bulinska-Radomska & Lester, 1985) intergradation is reported. Therefore some authors conclude that the cross-breeding species should not be considered as distinct species (Essad, 1954; Naylor, 1960; Bulinska-Radomska & Lester, 1985).

This paper presents an analysis of quantitative morphological and phenological data observed in several populations of different *Lolium* species. Previous publications have shown that multivariate analysis of quantitative characters can be used to measure genetic distances between populations within species (e.g. Souza & Sorrells, 1989; Humphreys, 1991). Although the phenotype based on quantitative characters cannot be directly related to the genotype, it has a strong genotypic basis. Therefore quantitative characters can be used as a measure of genetic distances between populations. In this study genetic distances between species are estimated on the basis of quantitative morphological and phenological characters, which are characters used in determination keys for the species. The results will be compared with results obtained by other authors using various techniques.

Material and methods

Plant material and experimental design

Fifty-one *Lolium* populations were obtained from several sources (gene banks, breeders, research institutes). These populations were classified into seven *Lolium* species, most of the time agreeing with the species name under which the seed was supplied (Table 1). If a population was classified as another species, it was decided on basis of seed morphology. In Table 1 the used code, cultivar name or population origin, seed source, species and compatibility group are tabulated for each population. A complete randomized block design with two blocks was used to analyse the populations. Each plot within a block consisted of two rows of ten plants, each plant spaced 37.5 cm apart within and between rows. Around the blocks a border row was planted. All populations were transplanted to the field on May 6, 1990. Populations belonging to *L. perenne* were vernalized. The field was situated in the CPRO-DLO experimental farm at Wageningen, on a sandy soil. The observed characters, the manner of scoring the characters and the time of observation are given in Table 2.

Data analysis

Analysis of variance was used to check for block and population effects. The mean and standard deviation of each trait was estimated for each species.

The characters were standardized across populations, and population averages were

Table 1: Code, cultivar name or population origin, seed source, species and compatibility group for all populations observed

Code	Cultivar (' ') or Population Origin	Seed source	Species	Compatibility
90.952	Hungary	CGN (G)	*L. perenne*	crossbreeder
10001	Turkey	CGN (G)		
90.955	Austria/Hungary	Van der Have (B)		
90.956	Austria/Hungary	Van der Have (B)		
90.957	Austria/Hungary	Van der Have (B)		
82.035	'Landrace'	Barenbrug (B)	*L. multiflorum*	crossbreeder
82.059	'Vitesse'	Van der Have (B)		
82.908	'Rocket'	Nickerson (B)		
83.946	'Menichetti'	Sisfiraggera (S)		
83.980	'Midmar'	D.P.S.C.(S)		
90.910	USA	Joordens (B)		
90.911	USA	Joordens (B)		
90.912	USA	Joordens (B)		
90.913	USA	Joordens (B)		
90.943	USA	Joordens (B)		
90.944	USA	Joordens (B)		
90.945	USA	Joordens (B)		
PI 277848*	Cyprus	White (S)		
90.946	USA	Joordens (B)		
90.953****	Turkey	CGN (G)		
90.905	'Wimmera'	Joordens (B)	*L. rigidum*	crossbreeder
90.906	selection Wimmera	Joordens (B)		
90.907	selection Wimmera	Joordens (B)		
90.908	selection Wimmera	Joordens (B)		
90.909	selection Wimmera	Joordens (B)		
90.914	South Australia	Kloot (S)		
90.915	Victoria	Kloot (S)		
90.916	Jabuk	Kloot (S)		
90.917	Reeves Plain	Kloot (S)		
90.919	Crete	Kloot (S)		
90.938**	Evia	CGN (G)		
90.942***	Japan	NIAR (G)		
90.920	Lazio	CGN (G)	*L. temulentum*	inbreeder
86.4885	Pakistan	CGN (G)		
86.4886	Pakistan	CGN (G)		
GRA 426/83	--	Gatersleben (G)	*L. remotum*	inbreeder
GRA 418/81	--	Gatersleben (G)		
PI 269386	Afghanistan	White (S)	*L. persicum*	inbreeder
PI 317450	Afghanistan	White (S)		
PI 163283	India	White (S)		
PI 222807	Iran	White (S)		
PI 229764	Iran	White (S)		
PI 230110	Iran	White (S)		
PI 239727	Iran	White (S)		
PI 239728	Iran	White (S)		
PI 239729	Iran	White (S)		
PI 314446	U.S.S.R	White (S)		
90.941	--	IGR-PAN (G)		
90.937	Peloponnesis	CGN (G)	*L. loliaceum*	inbreeder
90.939	North Crete	CGN (G)		
90.940	--	IGR-PAN (G)		

* received as L. persicum, classified as L. multiflorum, ** received as L. loliaceum, classified as L. rigidum, *** received as L. subulatum, classified as L. rigidum and **** received as L. perenne, classified as L. multiflorum. In parentheses: B=Breeding company, S=Scientist/Institute and G=Genebank

Table 2: Characteristics measured on fifty-one *Lolium* populations

char.	Description	Time of observation
1 DE	Date of ear emergence. Days after the first of May	1
2 NH	Natural height at ear emergence (cm)	1
3 H	Habitus. Scale 1=erect to 9=prostrate	1
4 LC	Leaf Colour. Scale 1=very light green to 9= dark green	1
5 AC	Anthocyanin in culms. Scale 1=very little to 9= dark red	1
6 LF	Length of the flag leaf (cm)	1
7 WF	Width of flag leaf(mm)	1
8 HA	Height 30 days after ear emergence (cm)	2
9 LNE	Length of the upper internode and ear (cm)	2
10 LN	Length of the upper internode (cm)	2
11 EL	Ear length (cm)	2
12 RL	Rachis length (mm)	2
13 NS	Number of spikelets per spike	2
14 LS	Length of spikelet awns not included (mm)	2
15 NF	Number of florets per spikelet	2
16 PA	Percentage awned florets	2
17 LA	Length longest awn (mm)	2
18 LG	Length of glume (mm)	2
19 SG	Spikelet length (14)/Length of glume (18)	2

Characteristics 14 to 18 were measured on a spikelet about half-way the spike.
Time of observation: 1= at ear emergence, 2= thirty days after ear emergence.

calculated for Principal Component Analysis (PCA, Chatfield & Collins, 1980). PCA will transform the original characters into a set of new, uncorrelated variables. These variables are arranged in order of decreasing importance, the first component being the most meaningful. Character redundancies are weighted out, because the analysis is a variable-dependent technique (also called R-technique) (Sneath & Sokal, 1973; Chatfield & Collins, 1980). When the scores of the populations on the first two components are plotted, independent grouping of the populations can be established.

The observations per plant were used for Canonical Variate Analysis (CVA, Chatfield & Collins, 1980), also a variable-dependent technique. Species and population were used as grouping variables, CVA optimizes the between group variation in relation to the within group variation. In the first CVA, the individual plants were grouped according to species. During CVA the squared Mahalanobis distance (D^2) is estimated each time a variable is entered in the analysis. If all variables are entered the final D^2 between the species is calculated. This distance was used to express the resemblance between the seven *Lolium* species. The second CVA was used to group according to population. The resulting canonical variate coefficients for each population on the first six axes were used for further analysis. The canonical variate coefficients can be considered as new, orthogonal, uncorrelated characters, which have the advantage over the original characters

Table 3: Mean (M) and Standard Deviation (SD) per character per *Lolium* species

	perenne		*multiflorum*		*rigidum*		*loliaceum*		*persicum*		*temulentum*		*remotum*	
	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
DE	58.9	14.4	46.3	14.8	45.6	11.6	36.5	15.7	22.1	7.2	22.5	10.1	31.2	4.6
NH	21.1	9.8	37.8	17.4	17.8	10.3	7.4	4.0	19.4	5.1	20.0	5.7	22.8	3.5
H	5.9	1.2	4.1	1.6	6.4	1.4	6.8	1.5	1.9	1.0	1.7	0.8	2.9	1.0
LC	5.8	0.7	5.3	0.7	5.4	0.8	5.5	0.7	4.1	0.7	4.0	0.7	4.6	0.5
AC	3.1	2.4	2.7	2.0	4.4	2.3	5.8	1.8	3.5	1.7	2.3	1.2	1.9	0.9
LF	17.9	6.4	19.2	8.7	11.3	5.8	7.2	4.0	9.7	2.5	8.9	2.5	7.5	3.4
WF	6.6	1.2	7.7	2.6	6.9	2.4	5.4	1.8	4.7	0.9	4.9	0.9	3.9	0.6
HA	73.7	12.4	104.8	17.2	81.0	17.3	26.2	13.1	46.9	11.1	49.4	14.5	54.6	8.6
LNE	52.6	9.3	65.6	10.0	52.8	9.2	19.5	8.7	38.0	8.8	39.7	12.4	41.8	4.6
LN	28.0	7.8	37.8	8.0	28.4	7.4	6.8	3.7	19.9	5.2	23.3	6.8	25.3	2.9
EL	24.5	5.4	27.9	5.6	24.5	5.6	12.7	5.7	18.7	4.7	16.4	6.2	16.5	3.2
RL	2.6	0.6	3.1	0.7	2.3	0.5	1.3	0.6	2.7	0.9	2.3	1.0	2.7	0.7
NS	22.2	6.1	21.5	5.3	20.9	5.4	16.3	2.7	11.6	2.2	11.5	2.2	13.6	1.9
LS	1.9	0.3	2.1	0.4	2.0	0.3	1.2	0.4	1.7	0.5	1.5	0.5	1.5	0.3
NF	12.9	2.2	14.0	3.1	10.7	1.8	6.8	2.0	6.1	1.5	6.4	1.9	10.2	2.1
PA	0.1	1.1	55.2	33.0	2.6	9.8	0.0	0.0	99.8	2.2	99.3	5.6	46.3	27.4
LA	0.0	0.1	3.2	2.2	0.2	1.5	0.0	0.0	9.6	3.3	9.4	2.0	1.1	0.7
LG	1.2	0.2	1.0	0.3	1.4	0.3	1.4	0.4	1.4	0.4	1.4	0.4	1.1	0.2
SG	1.6	0.3	2.1	0.5	1.5	0.3	0.8	0.1	1.2	0.3	1.0	0.2	1.3	0.2

Character abbreviations see table 2. M mean, SD standard deviation

that they do not bias the results due to correlation of characters. The coefficients were used to cluster the populations. The measure of distance between the populations was the Squared Euclidian Distance: $S(x,y)=\Sigma_i(x_i-y_i)^2$. The populations were clustered using Ward's method (Dreichsel & Trampisch, 1985). This method clusters according to the criterion that the sum of the weighted centroid distances of both clusters to the centroid of the potential new cluster must be as small as possible. The clustering of the populations was terminated at the seven group level, which agrees with the number of species entered in the analysis. The mean and standard deviation for each character was calculated, using the original data, for the resulting seven cluster groups.

Results

Analysis of variance indicated a significant population effect ($P \leq 0.001$) for all characters. Block effect however was not significant for any character. In Table 3 the estimated means and standard deviations for all characters of each species are tabulated. These figures are based on a minimum of two populations per species.

Principal Component Analysis (PCA)

The eigenvalues of, the percentage of variation explained by, and the five variables with the highest Principal Component loadings on the first three Principal Components are tabulated in Table 4. The plot of the populations on the first two Principal Components is shown in Figure 1. These two axes explain 78.54% of the observed variation.

Table 4: First three Principal Components: eigenvalues, percentage of variance explained and the original characters with the highest loadings on the first three Principal Components. Based on fifty-one *Lolium* populations.

Principal Component		1		2		3
Latent root		357.2		153.72		57.42
Percent of variance		54.91		23.63		8.83
Principal Component scores	HA	0.34	PA	-0.49	LG	-0.51
	LNE	0.31	H	0.43	LS	-0.49
	NF	0.29	LA	-0.42	NS	-0.31
	DE	0.28	NH	-0.27	LF	0.27
	EL	0.28	EL	-0.25	NH	0.24

Character abbreviations see table 2

Figure 1 shows that populations assigned to the same species are generally grouped together. The cross- and inbreeding species are well separated, with *L. remotum* populations in an intermediate position between the two groups and the *L. loliaceum* populations rather isolated. The populations of *L. temulentum* and *L. persicum* overlap. Within the cross-breeding group *L. multiflorum* is close to but separate from *L. perenne* and *L. rigidum*. The separation of *L. perenne* and *L. rigidum* is not clear as one *L. perenne* population (of five) is located within the group of *L. rigidum* populations.

Table 4 combined with Figure 1 shows that the first Principal Component separates the populations based on plant size (HA, LNE and EL), number of florets (NF) and earliness (DE). The populations oriented in the left half of the scatterplot are generally smaller, contain fewer florets and the ears emerge earlier. The populations oriented in the right half of the scatterplot are taller, contain more florets per spikelet and the ears emerge later. The second Principal Component separates the populations with more (PA) and longer awns (LA), more erect (H) and taller (NH and EL) plants (in the lower half of the scatterplot) from these with fewer and smaller awns, more prostrate and smaller plants (upper half of the scatterplot).

Canonical Variate Analysis (CVA)

The squared Mahalanobis distances (D^2) between the *Lolium* species are tabulated in Table 5. The smallest D^2 was found between *L. persicum* and *L. temulentum*, the largest between *L. perenne* and *L. persicum*. The smallest squared Mahalanobis distance between a cross-breeder and an inbreeder was found for *L. rigidum* and *L. loliaceum*. In general the distances between the cross-breeding species were smaller than the distances between the inbreeding species.

The first six canonical variate axes explained 93.11 % of the observed variation, all six explained a significant part of the observed variation. The phenogram obtained after clustering, based on the canonical variate coefficients of each population on these six axes, is given in Figure 2.

This phenogram shows that when clustering of the populations was terminated at the seven group level, the groups generally agreed with the species designation of the populations. Two populations appeared to be misclassified (e.g. *L. multiflorum* Cyprus ; *L. perenne*

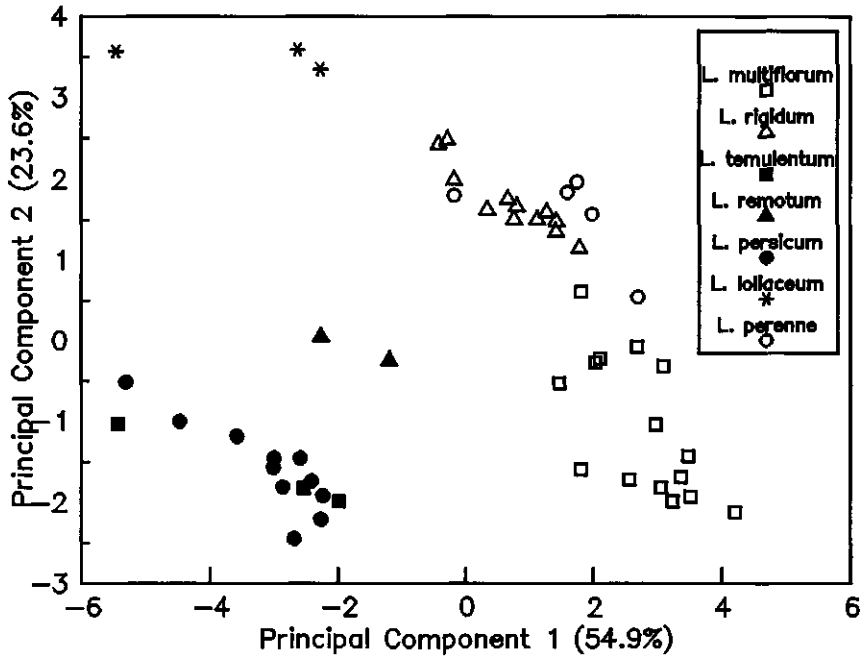

Figure 1: Scatterplot of 51 *Lolium* populations on the first two principal components

Turkey). The populations of *L. temulentum* and *L. persicum* were divided over two clusters (cluster 6 and 7, Figure 2), both clusters containing populations from both species. These two cluster groups were clearly distinct from the other cluster groups. *L. loliaceum* and *L. remotum* were clustered closer to the cross-breeding species than to the other two inbreeding species, *L. temulentum* and *L. persicum*.

As most of the cluster groups generally agreed with one species, the means for each character

Table 5: The squared Mahalanobis distances between seven *Lolium* species

Species	1	2	3	4	5	6	7
1 *L. perenne*	0.00	4.95	3.61	6.12	9.43	9.15	5.40
2 *L. multiflorum*		0.00	4.78	8.45	8.43	8.17	5.28
3 *L. rigidum*			0.00	5.23	8.28	8.09	5.39
4 *L. loliaceum*				0.00	8.09	7.86	6.17
5 *L. persicum*					0.00	1.78	6.41
6 *L. temulentum*						0.00	5.80
7 *L. remotum*							0.00

were very alike. Only cluster 6 and cluster 7 showed large deviations from the means of the species they are composed of. Cluster six contains the earlier, more erect, less tall, fewer florets bearing and shorter awned populations of both species. Cluster 7 contains the later, less erect, taller, more florets bearing and longer awned populations of both species.

Discussion

The extensive literature on the species in the genus *Lolium* is difficult to summarize. Terrell (1966) states that material used for crossing experiments often is of obscure origin. Another restraint is that most experiments have been done with only a very limited number of genotypes, so that results largely depend on the combining ability of those genotypes. The results of crossing experiments should therefore be treated with care. Information like seed protein banding pattern and DNA-amount is more reliable, but again data are only available for a very limited number of populations and plants per population. Another problem arises with the determination of the C-banding pattern of chromosomes. Only one report (Thomas, 1981) is known on the C-banding patterns of the several *Lolium* spp. Several authors report (e.g. Linde-Laursen et al., 1986) variation in C-banding pattern between homologous chromosomes within one cell, variation between chromosomes of different cells in one plant and variation between plants of one population. This could also be the case for *Lolium* populations, and as the C-banding patterns reported by Thomas (1981) are based on one population per species, the usefulness of this information is limited. Morphological data are numerous and are often measured on large samples of populations. The restraint of these data is that it is not always stated clearly how the data were obtained and analyzed. Also different statistical techniques can give different results, which makes comparison difficult. Furthermore the relative importance of the results of different methods is arbitrary. It is obvious that in the present study populations from seven *Lolium* species are compared in one field trial, enhancing the comparison of the morphological variation found. The genotype-environment interaction cannot be determined with this trial.

Distinction between cross-breeding and inbreeding species

The results from this trial indicate that the cross-breeders are easily separated from the inbreeding species. Two of the inbreeding species (*L. temulentum* and *L. persicum*) are clearly distinct from the cross-breeding species. The other two, *L. loliaceum* and *L. remotum*, appear more closely related to the cross-breeding group (figs. 1, 2). The cross-breeders are separated from the inbreeders primarily by the first Principal Component (Figure 1). This indicates that cross-breeding *Lolium* populations can be best separated from inbreeding populations by plant size (height after ear emergence (HA), length of the upper internode and ear (LNE) and ear length (EL)), number of florets (NF) and earliness (DE). In general the inbreeding populations are smaller, contain fewer florets and the ears emerge earlier (Table 3 and 4).

On the basis of squared Mahalanobis distances between species, *L. remotum* appears to be closer to the cross-breeding species than

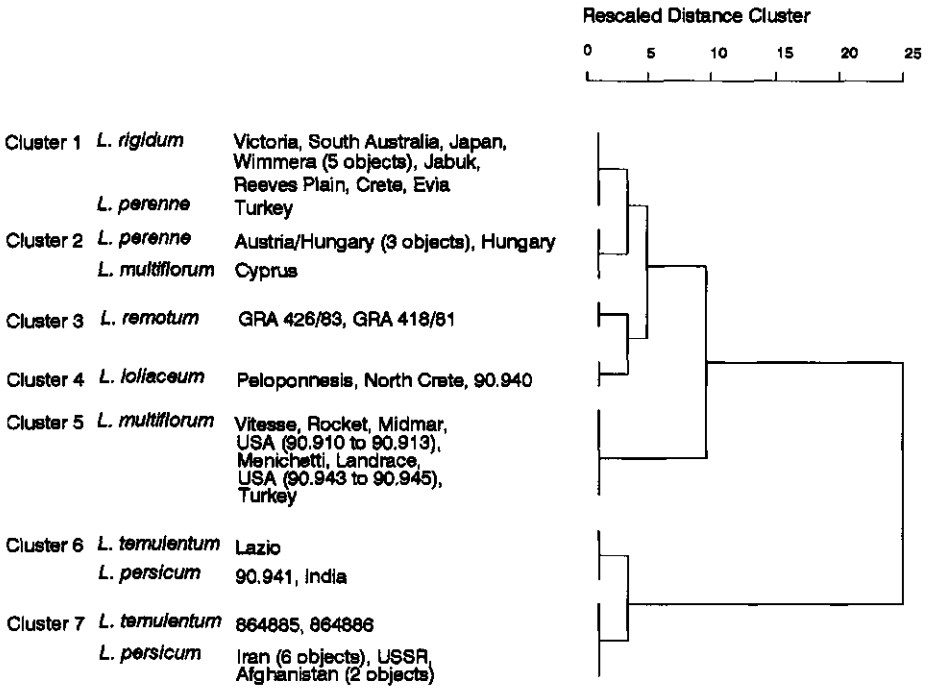

Figure 2: Phenogram based on the first six canonical variate scores of 51 *Lolium* populations, clustered using Ward's method

suggested in any other study. Although the smallest distance was found between *L. remotum* and *L. multiflorum*, the distances to the other two cross-breeding species (*L. perenne* and *L. rigidum*) are only slightly larger. This close relationship between *L. remotum* and the cross-breeding species can also be seen in PCA (Figure 1) and in clustering (Figure 2). The DNA content of *L. remotum* is larger than that of any of the cross-breeding species and the C-banding pattern of the chromosomes is also different (Thomas, 1981). In a study on morphological and protein variation of several *Lolium* spp. by Bulinska-Radomska & Lester (1985), *L. remotum* populations are morphologically very similar to some *L. rigidum* populations. But the similarity of protein spectra is very low between *L. remotum* and the cross-breeding species, even lower than that of *L. temulentum* and the cross-breeding species. Crossing experiments (Naylor, 1960) do not indicate that crosses between *L. remotum* and the cross-breeding species are more successful than crosses of the other inbreeding species with the cross-breeding species. Hutchinson (1979)

reports better fertility of the F_1 hybrids of *L. remotum* and *L. rigidum* than the F_1 hybrids of *L. remotum* with the other two cross-breeding species. So no confirmation can be found, in literature, that *L. remotum* is intermediate between the cross-breeding and inbreeding species, as indicated by the present results.

The squared Mahalanobis distance between *L. loliaceum* and *L. rigidum* is the smallest found between an inbreeder and a cross-breeder. In the scatterplot (Figure 1) the *L. loliaceum* populations have a rather isolated position: they are clustered into their own cluster (Fig. 2) close to the cross-breeding species. Thomas (1981) suggested that *L. loliaceum* should be considered as intermediate between the cross-breeding and the inbreeding group, based on the intermediate DNA-amount of this species and the great similarity of the C-banding pattern of this species with that of the cross-breeding species. Crosses (Naylor, 1960) between *L. loliaceum* and the cross-breeding species are not more successful than crosses of the other inbreeding species with cross-breeding species. The results from the present study agree with Thomas's (1981) view that *L. loliaceum* is a species distinct from the other inbreeders, but its position is not intermediate but rather isolated and closest to *L. rigidum*.

L. temulentum and *L. persicum* are clearly distinct from the cross-breeding species (Figure 1). All Mahalanobis distances are the largest found (Table 5), and during clustering the populations of these two species are joined with the other populations in the last step (Figure 2). The DNA-amount of both inbreeding species is clearly much larger (about 40%) than that of the cross-breeding species, the C-banding pattern of the chromosomes of *L. temulentum* is very distinct (Thomas, 1981). Bulinska-Radomska & Lester (1985) reported low similarities between the protein spectra of *L. temulentum* and the cross-breeding species. Crosses (Naylor, 1960) between *L. temulentum* and the cross-breeding species give poor results. However, Hutchinson (1979) found that the hybrids between *L. rigidum* and *L. temulentum* are more fertile than the hybrids of *L. temulentum* with the other two cross-breeding species. For *L. persicum* only information on the DNA-amount is available, other experimental results for this species were not reported. Both the PCA (Fig. 1) and the CVA (Tab. 5, Fig. 2) agree with literature that both species are clearly distinct from the rest of the genus.

Distinction between cross-breeding species

The three cross-breeding species are very similar. The squared Mahalanobis distances between the three species are among the smallest found among the several *Lolium* species (Table 5). The plot of the first two Principal Components (Fig. 1) shows that the populations are located in the same half of the plot, the populations seem to be grouped according to species but do intergrade. The first Principal Component does not separate the populations of the three species clearly. On this Principal Component the populations are mainly separated due to plant size (HA, LNE and EL) and number of florets (NF). On one end the smaller and fewer florets containing *L. rigidum* populations are found and on the other end the taller more florets containing *L. multiflorum* populations are found (Table 3 and 4). The *L. perenne* populations appear to be

intermediate. The second Principal Component separates the *L. multiflorum* populations from the populations of the other two species on basis of the presence (PA) and length of the awns (LA), the more erect habitus (H) and the plant size (NH and EL) (Table 3 and 4). The *L. perenne* and *L. rigidum* populations are not separated clearly by this second Principal Component, due to one *L. perenne* population which is located within the *L. rigidum* group. After clustering, the cross-breeding populations are divided over three cluster groups (cluster 1, 2 and 5, Figure 2) corresponding with the species groups. *L. perenne* and *L. rigidum* appear to be the most similar species within the cross-breeding group (Table 5, Fig. 2). Bulinska-Radomska & Lester (1985) found that on basis of protein similarities *L. multiflorum* and *L. rigidum* are more closely related than any other combination of cross-breeding species. The karyomorphology of the three species is also very similar, although they differ in DNA-amount. The C-banding pattern of the chromosomes of *L. perenne* and *L. rigidum* is identical (Thomas, 1981). The crosses between the three cross-breeding species are all equally successful (e.g. Terrell, 1966; Hutchinson, 1979), and do not indicate a closer relationship between any pair of the cross-breeding species.

Some authors prefer to consider the cross-breeding species as one species. This seems a rash conclusion as the species do show morphological and chromosomal differences. Crawford (1983) suggested three possible explanations for morphological intergradation of species:

- the taxa are of recent origin and have not yet diverged far,
- taxa do have different genomes but plasticity makes them look alike under the same environmental conditions,
- interspecific hybridisation.

The second explanation does not seem to apply for *Lolium*, although studies have shown that *Lolium* populations do adapt very rapidly to different environments. But the genomes of the three cross-breeding species are much alike (Thomas, 1981; Hutchinson, 1979) and as shown in the present study under the same environmental conditions morphological differences are present. Hybridisation is also not likely (although interspecific crossing is well possible), because the two species most similar morphologically and karyotypical *L. perenne* and *L. rigidum* (Thomas, 1981), hardly have overlapping distribution areas. The first explanation seems the most plausible; some authors state that speciation in *Lolium* is of recent origin (Malik, 1967; Darlington & Mather, 1949).

Distinction between inbreeding species

The second Principal Component appears to be most successful in separating the populations of the different inbreeding *Lolium* species. This Principal Component separates the small, unawned and prostrate growing *L. loliaceum* populations very clearly from the taller (NH and EL), awned (PA and LA), erect growing (H) populations of *L. persicum* and *L. temulentum* (Table 3 and 4). The *L. remotum* populations are intermediate as they are taller and grow more erect like *L. persicum* and *L. temulentum*, but are largely unawned and the awns present are very small (Table 3).

The squared Mahalanobis distance between *L. persicum* and *L. temulentum* is the smallest found (Table 5). Both in the plot (Fig. 1) and

the phenogram (Fig. 2) *L. temulentum* and *L. persicum* populations could not be separated. The morphological similarity of both species can be well explained by the fact that both species are known as weeds in cereals (Terrell, 1968; Dore, 1950), thus selection might have modified them into the same direction. Dore (1950) states that when *L. persicum* was first introduced in Canada it was classified as *L. temulentum* because of its great similarity. On basis of this trial it was impossible to recognize them as species. This should not be interpreted as that the cluster groups can be considered as new species groups, as the results are based on a limited number of characters and populations. The use of seed characters could enhance the distinction of the populations of both species in this trial, as seed characters are often used as a determination characters for *L. persicum* and *L. temulentum*(Terrell, 1968). Furthermore Thomas (1981) and Hutchinson (1979) report a slight difference in DNA amount between both species. DNA amount is in general very constant within species (Rees & Jones 1974). This supports the view that the two species should be recognized.

L. remotum and *L. temulentum* are very closely related species, and only differ as they evolved as weeds in different crops (Jenkin, 1954; Naylor, 1960; Terrell, 1966). On basis of the Mahalanobis distance (Table 5) *L. remotum* is indeed closest to *L. temulentum*. The DNA-content of *L. remotum* is intermediate between *L. temulentum* and *L. loliaceum* and the C-banding pattern of *L. remotum* is very similar to that of *L. temulentum* (Thomas, 1981). Terrell (1966) reports that the crosses between *L. remotum* and *L. loliaceum* are less successful than the crosses between *L. remotum* and *L. temulentum*, this because the former crosses yield more abnormal F_1 plants. While the foregoing suggests that *L. remotum* is more closely related to *L. temulentum*, in the phenogram (Fig. 2) *L. remotum* is clearly more similar to *L. loliaceum*. This is mainly caused by the fact that percentage awns (PA) and length of the awn (LA) are highly correlated with each of the six canonical variate axes, of which the scores were used for clustering. The awned populations are therefore clearly separated from the less awned and unawned populations in the phenogram (Table 3, Fig. 2).

Conclusion

Although there are many problems with the comparison of all the data available for the different *Lolium* species, the following can be concluded. It appears that the genus *Lolium* can be divided into two groups. An inbreeding group, containing *L. persicum* and *L. temulentum*, clearly distinct from the rest of the genus. Within this group it is doubtful whether two species can be recognized. A second group contains all three cross-breeding species (*L. perenne*, *L. multiflorum* and *L. rigidum*); these species are closely related but seem to be distinct, although introgression between all three species is well possible. The two remaining inbreeding species (*L. loliaceum* and *L. remotum*) do not form a distinct group. *L. remotum* is intermediate between the cross-breeding species and the other inbreeding species, and *L. loliaceum* is in a somewhat isolated position, more closely related to *L. rigidum*.

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CHAPTER 3:

ALLOZYME VARIATION WITHIN- AND BETWEEN-POPULATIONS IN *LOLIUM* (*POACEAE*)

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Summary

Isozyme analysis was used to determine genetic variation within and between populations of seven *Lolium* species. All populations from the inbreeding species (*L. temulentum*, *L. remotum*, *L. loliaceum* and *L. persicum*) were completely fixed for all enzymes scored. They also contained, for four of the five enzyme systems studied, exactly the same allelic variant. The three cross-breeding species showed large within-population variation and much less between-population variation. The great similarity of the allozymic variants found in all species, made the division of the genus *Lolium* into species on basis of allozymic data difficult. It was not possible to separate the different inbreeding species from each other. Within the cross-breeding group *L. multiflorum* and *L. rigidum* could be distinguished from *L. perenne*. *L. multiflorum* and *L. rigidum* could, with more difficulty, also be separated from each other.

Allelic variation could have more relation with the provenance of the populations than with taxonomic classification.

Introduction

In the genus *Lolium* (*Poaceae*) genetic variation is often assessed by measuring morphological characters. The disadvantage of many of these characters is, that they are influenced by environmental factors. It can be argued that these characters measure the adaptation of a population to a habitat, instead of being an objective measure of genetic variation. The genetic basis of the morphological characters is often oligo- or polygenetic, which makes the direct translation of phenotype into genotype impossible, although there is clearly a relation. Other techniques for measuring genetic variation, used in the genus *Lolium*, are: karyotypic analysis, DNA-content determinations and C-banding pattern assessment (Malik & Thomas, 1966; Thomas, 1981), and assessment of biochemical characteristics (Bulinska-Radomska, 1985). Again, the variation found cannot be related to the genotype directly.

Electrophoresis is often used to assess the genetic variation of populations for distinct allelic forms of isozymes. The main advantages are that isozymes are unlinked characters often hardly or not environmentally influenced and, since many isozymes show codominance, identification of heterozygotes is possible. A disadvantage is that it is not known whether or not isozymes show adaptive response. In the case of *Lolium perenne* L. (*Poaceae*). Rainey et al. (1987) indicate that there is a correlation between certain allelic variants of Phosphoglucose-mutase (PGM) and 6-phosphogluconate-dehydrogenase (6-PGD) and the photosynthesis efficiency. Another constraint is that the scored isozymes in a study are only a small

sample of the total available protein variation, obtained by the use of only one technique (Stebbins, 1990). Isozyme data can be used for several types of research. For instance, similarity of species and populations can be established (e.g. Novak, 1989) and evidence for chromosomal evolution like gene duplication can be found (Gottlieb, 1983).

In this study the objective was to analyse the genetic variation for several isozymes in populations of seven *Lolium* species: *L. perenne* L., *L. multiflorum* Lam., *L. rigidum* Gaud., *L. loliaceum* Hand.-Mazz., *L. temulentum* L., *L. persicum* Boiss. & Hohen. ex Boiss. and *L. remotum* Schrank. The first three are cross-breeding and the latter four are inbreeding species. A second objective is to determine whether the found allelic forms and variation have any affiliation with the taxonomic classification of the populations. For *Lolium* species only studies on the isozymic variation of *L. perenne* and *L. multiflorum* are known. Østergaard et al. (1985) analysed several *L. perenne* and *L. multiflorum* cultivars for five isozymes, with the objective to distinguish the species and the cultivars. Other authors also use isozymes with the purpose to distinguish between *Lolium* cultivars (e.g. Nielsen et al. 1985, Lallemand et al. 1991). All authors do report differences in isozyme pattern between the cultivars and species.
A number of the populations in this study were also used in measuring variation in morphological characters (Loos in press), the results from both studies will be compared.

Table 1: Enzyme systems used, locus abbreviation, number of alleles observed for the electrophoretic examination of twenty-five *Lolium* populations.

Enzyme and EC number	Abbreviation locus	Number of alleles
Acid phosphatase [EC 3.1.3.2]	Acp	5
Phosphoglucoisomerase [EC 5.3.1.9]	Pgi-2	7
Phosphoglucomutase [EC 2.7.5.1]	Pgm	4
Shikimate dehydrogenase [EC 1.1.1.25]	Sdh	4
6-Phosphogluconate dehydrogenase [EC 1.1.1.44]	6-Pgd	3

Material and methods

Five allozymes were used for electrophoretic analysis of twenty-five populations divided over seven *Lolium* species. The used allozymes and the number of alleles found for each locus are summed in Table 1.

Plant material

For each population leaves of 60 four-weeks old seedlings were analysed. The seedlings were grown in a Conviron growth chamber: 16 h light - 8 h dark, at a constant temperature of 15 °C. The code, cultivar name or population origin, seed source, species and breeding system are given for each population in Table 2.

Electrophoretic procedures

Seedlings were ground in 35 μl extraction buffer (0.01M Tris/HCl, pH 7.15, 3 mM DTE, 5% sucrose). Wicks were soaked in 15 μl of the leaf extracts and loaded on a 12 by

Table 2: The seven *Lolium* spp.: the code, cultivar or population origin, seedsource, species and compatibility for each population examined

Code	Cultivar (' ') or Population origin	Seedsource	Species, Compatibility
90.956	Austria/Hungary	Van der Have (B)	*L. perenne*
10001	Turkey	CGN (G)	cross-breeder
09957	Netherlands	CGN (G)	
864776	Czechoslovakia	CGN (G)	
82.035	'Landrace'	Barenbrug (B)	*L. multiflorum*
82.059	'Vitesse'	Van der Have (B)	cross-breeder
82.908	'Rocket'	Nickerson (B)	
83.946	'Menichetti'	Sisfiraggera (S)	
90.913	USA	Joordens (B)	
PI 277848*	Cyprus	White (S)	
90.905	'Wimmera'	Joordens (B)	*L. rigidum*
90.914	South Australia	Kloot (S)	cross-breeder
90.916	Jabuk	Kloot (S)	
90.919	Crete	Kloot (S)	
90.938	Evia	Kloot (S)	
864885	Pakistan	CGN (G)	*L.temulentum*
864886	Pakistan	CGN (G)	inbreeder
GRA 426/83	--	Gatersleben (G)	*L. remotum*
GRA 418/81	--	Gatersleben (G)	inbreeder
PI 269386	Afghanistan	White (S)	*L. persicum*
PI 163283	India	White (S)	inbreeder
PI 229764	Iran	White (S)	
PI 314446	ex-USSR	White (S)	
90.937	Peloponnesis	CGN (G)	*L. loliaceum*
90.939	North Crete	CGN (G)	inbreeder

* received as *L. persicum*, classified as *L. multiflorum*. In parenthesis: B= breeding company, S=Scientist/institute and G= genebank.

19 cm gel, made of 350 ml gel buffer, 11% starch and 15 gr sucrose. The gel and electrode buffer were made from the following components: gel buffer 0.010 M histidine, electrode buffer 0.135 M Tris and 0.043 M citric acid. Both buffers had a pH of 7.0 titrated with a 1 M citric acid solution. Gels were run under 33.3 V/cm and at a maximum of 75 mA for 2.3 hours. Brome cresol green was used as marker dye. An inbreeding population, *L. temulentum* (864886, see Table 2), was used as allelic marker. On each gel three individuals from this population were run as a reference for the position of the alleles on the gel. The gel was sliced and stained following the staining procedures, for all five enzymes, of

Wendel & Weeden (1990). All enzymes migrated towards the anode. For each allozyme the alleles were alphabetically designated, with the most anodal form being "a".

Data analysis

The gene frequencies were analysed using BIOSYS-1 (Selander & Sowford, 1983). The data for all allozymes were entered per population. To measure the genetic variation of populations the mean number of alleles per locus (A), the direct-count heterozygosity across all loci (H_{obs}), the expected heterozygosity under Hardy-Weinberg equilibrium across all loci (H_{exp}) and Wright's fixation index, $F = 1 - H_{obs}/H_{exp}$, were calculated.

Nei's gene diversity statistics (1973, 1978) were used to divide the total allelic variation within each species. H_T represents the total allelic variation at a locus within each species. This H_T can vary from zero, indicating that there is no variation, to one (maximal variation). The H_T is split up in H_S, mean allelic diversity within populations, and D_{ST}, mean allelic variation between populations. The relation between these three parameters is that $H_T = H_S + D_{ST}$. A fourth parameter, G_{ST}, is calculated as D_{ST}/H_T, and represents the proportion of the total allelic variation found between the populations.

All four parameters were calculated per compatibility group for each locus, using only the variable species and taking the number of populations into account.

Nei's genetic identity (I, Nei, 1972) was calculated for all combinations of populations and averaged for each species to give an indication of the identity of species. This identity (I) can vary from zero (no alleles in common) to one (identical allelic composition and frequencies) for two populations.

The frequencies of each allele found were used as variables in the data matrix that was used for multivariate analysis. This matrix contained 25 objects (populations) with 23 variables (alleles) each (Table 3), the frequencies were transformed using the arcsin transformation to reduce skewness. Principal Component Analysis (PCA, Chatfield & Collins, 1980) was performed to establish which alleles expained the most of the observed variation. The scores of the populations on the first three axes were used to determine independent grouping of the populations.

Results

In Table 4 the average number of alleles per population (A), the observed and expected heterozygosity (H_{obs} and H_{exp}) and Wright's fixation index are tabulated for each population. The average number of alleles of the cross-breeding species (A= 1.8 - 3.6) was larger than that of the inbreeding species (A=1.0 - 1.2). Within the cross-breeding group *L. perenne* populations had less alleles per locus (A= 1.8 - 2.4) than the populations of *L. rigidum* and *L. multiflorum* (A= 2.8 - 4.0).

The H_{obs} and H_{exp} were larger for the cross-breeding populations than for the inbreeding populations. These parameters generally had a value of zero for the inbreeding species. Therefore the inbreeding populations were all completely fixed (F=1.00), the cross-breeding populations were more or less in equilibrium. Some population-unique alleles were detected, but no species could be characterized.

Nei's gene diversity statistics are presented in Table 5. For all loci examined the H_T of

Table 3: Allelic frequencies summarized for 5 isozyme loci for 25 *Lolium* populations.

Population	Acp a	Acp b	Acp c	Acp d	Acp x	Pgi a	Pgi b	Pgi c	Pgi c*	Pgi d	Pgi e	Pgi x	Pgm a	Pgm b	Pgm c	Pgm d	Sdh a	Sdh b	Sdh c	Sdh x	Pgd a	Pgd b	Pdg c
Lp 90.956	29.7	60.9	9.4	0	0	32.8	67.2	0	0	0	0	0	48.4	51.6	0	0	0	100	0	0	0	100	0
Lp 10001	34.4	28.1	0	25.0	12.5	11.3	9.7	9.7	0	69.4	0	0	79.7	20.3	0	0	0	100	0	0	0	100	0
Lp 09957	96.9	3.1	0	0	0	43.9	50.0	4.5	0	1.5	0	0	95.3	4.7	0	0	4.7	95.3	0	0	0	100	0
Lp 864776	39.1	48.4	6.3	6.3	0	35.9	56.3	0	7.8	0	0	0	50.0	50.0	0	0	0	100	0	0	0	100	0
Lm 82.035	13.3	76.6	2.3	7.8	0	3.9	27.3	17.2	0.8	50.8	0	0	78.1	19.5	2.3	0	8.1	55.6	36.3	0	1.6	98.4	0
Lm 82.059	12.9	73.4	7.3	6.5	0	4.0	40.3	21.0	0	34.7	0	0	57.9	40.5	1.6	0	11.1	33.3	55.6	0	0	100	0
Lm 82.908	12.9	81.8	0.8	4.5	0	20.0	21.5	17.7	1.5	39.2	0	0	69.8	29.4	0.8	0	6.5	63.7	29.8	0	0	100	0
Lm 83.946	24.1	63.0	0.9	12.0	0	11.1	33.3	9.3	2.8	42.6	0.9	0	87.7	11.3	0.9	0	6.9	76.5	15.7	0.9	4.4	91.1	4.4
Lm 90.913	1.8	89.5	0.9	7.9	0	19.7	13.9	4.9	0	61.5	0	0	98.2	1.8	0	0	0	31.9	68.1	0	0	98.2	1.8
Lm PI 277848	42.2	50.0	6.3	1.6	0	12.5	46.9	4.7	3.1	32.8	0	0	71.0	29.0	0	0	1.6	70.3	28.1	0	0	100	0
Lr 90.905	3.2	67.7	28.2	0.8	0	6.3	14.8	3.1	3.1	64.1	8.6	0	84.9	14.3	0.8	0	6.0	94.0	0	0	0	99.2	0.8
Lr 90.914	10.2	68.6	6.8	14.4	0	15.6	35.9	0.8	5.5	32.0	7.0	3.1	84.9	14.3	0.8	0	6.0	94.0	0	0	0	100	0
Lr 90.916	38.9	24.6	6.3	30.2	0	8.2	42.6	0	0	29.5	19.7	0	71.8	12.9	15.3	0	0	84.8	15.2	0	0	100	0
Lr 90.919	1.6	74.2	8.1	16.1	0	9.4	58.7	2.9	0	29.0	0	0	72.4	14.9	9.0	3.7	25.8	68.7	4.5	0	0.8	99.2	0
Lr 90.938	4.0	65.3	3.2	27.4	0	2.4	28.2	1.6	4.8	58.1	4.8	0	96.0	4.0	0	0	22.6	71.8	5.6	0	0	100	0
Lt 864885	0	100	0	0	0	0	0	100	0	0	0	0	100	0	0	0	0	100	0	0	0	100	0
Lt* 864886	0	100	0	0	0	100	0	0	0	0	0	0	100	0	0	0	0	100	0	0	0	100	0
Lre GRA426/83	0	100	0	0	0	0	100	0	0	0	0	0	100	0	0	0	0	100	0	0	0	100	0
Lre GRA418/81	0	100	0	0	0	0	98.4	1.6	0	0	0	0	100	0	0	0	0	100	0	0	0	100	0
Lps PI269386	0	100	0	0	0	0	0	100	0	0	0	0	100	0	0	0	0	100	0	0	0	100	0
Lps PI163283	0	100	0	0	0	0	0	0	100	0	0	0	100	0	0	0	0	100	0	0	0	100	0
Lps PI229764	0	100	0	0	0	0	0	0	0	100	0	0	100	0	0	0	0	100	0	0	0	100	0
Lps PI314446	0	100	0	0	0	0	0	100	0	0	0	0	100	0	0	0	0	100	0	0	0	100	0
Ll 90.937	0	100	0	0	0	0	0	0	0	100	0	0	100	0	0	0	0	100	0	0	0	100	0
Ll 90.939	0	100	0	0	0	0	0	0	0	100	0	0	100	0	0	0	0	100	0	0	0	100	0

Lp= *L. perenne*, Lm= *L. multiflorum*, Lr= *L. rigidum*, Lt= *L. temulentum*, Lre= *L. remotum*, Lps= *L. persicum* and Ll= *L. loliaceum*. For the population numbers see table 2. Lt*= population used as allelic marker during each electrophoresis run. 'x' allele= allele more anodal than the 'a' allele only occurring in few populations and in low frequencies. For the interpretation of Pgi, more specific for the 'c' and 'c*' allele (Loos & Degenaars, 1993).

the cross-breeding species was the largest. For the cross-breeding species most variation was found within populations, Pgi-2 was the most variable locus. The inbreeding species only showed variation at the Pgi-2 locus, with nearly all variation being distributed between populations. Only one inbreeding population showedwithin-population variation.

The mean genetic identities of the different *Lolium* species are summarized in Table 6. For all species, except *L. temulentum*, the genetic identity between populations of the same species was higher than the genetic identity between populations of different species. All genetic identities were relatively high, indicating high similarity of the different *Lolium* species. *L. multiflorum* and *L. rigidum* had the highest genetic identity (0.915). The overall lowest genetic identity was found for *L. perenne* and *L. persicum* (0.780). Within the inbreeding group the identities were almost identical (ca. 0.800). Only *L. loliaceum* and *L. persicum*, and *L. persicum* and *L. temulentum* had a higher identity. All inbreeders showed the highest identity with *L. rigidum*, second highest identity with *L. multiflorum* and lowest identity with *L. perenne*. *L. remotum* had a higher genetic identity with all the cross-breeding species than with the inbreeding species.

The scores of all populations on the first two Principal Components are plotted in a scatterplot (Figure 1). The percentage variation explained and the five variables with the highest loadings on the first three Principal Components are tabulated in Table 7. In this scatterplot it can be seen that there are some difficulties in separating the populations of *L. multiflorum* and *L. rigidum*, and the populations of *L. persicum* and *L. temulentum*. On

Table 4: Genetic variation in 25 *Lolium* populations

Population	Crossbreeding species	A	H_{obs}	H_{exp}	F
Austria/Hungary	*L. perenne*	1.8	.292	.299	.023
Turkey	*L. perenne*	2.4	.246	.312	.211
Netherlands	*L. perenne*	2.2	.165	.161	-.025
Czechoslovakia	*L. perenne*	2.2	.338	.336	-.005
'Landrace'	*L. multiflorum*	3.4	.385	.395	.025
'Vitesse'	*L. multiflorum*	3.0	.423	.439	.036
'Rocket'	*L. multiflorum*	3.2	.396	.396	.000
'Menichetti'	*L. multiflorum*	4.0	.339	.401	.115
USA	*L. multiflorum*	2.8	.260	.254	-.024
Cyprus	*L. multiflorum*	3.0	.378	.419	.098
'Wimmera'	*L. rigidum*	3.4	.239	.283	.155
South Australia	*L. rigidum*	3.6	.312	.326	.043
Jabuk	*L. rigidum*	2.8	.406	.420	.033
Crete	*L. rigidum*	3.4	.399	.380	-.050
Evia	*L. rigidum*	3.2	.326	.319	-.020
Population	Inbreeding species	A	H_{obs}	H_{exp}	F
GRA 418/81	*L. remotum*	1.2	.006	.006	1.000
All other inbreeding populations		1.0	.000	.000	1.000

A= mean number of alleles across loci, H_{obs}= Direct-count heterozygosity across all loci, H_{exp}= under Hardy-Weinberg expected heterozygosity. F= Wright's fixation index across all loci within each population.

Table 5: Nei's gene diversity statistics summarized (Nei, 1973 & 1978) for the cross- and inbreeding group of the genus *Lolium* for each locus.

Locus	group	H_T	H_S	D_{ST}	G_{ST}
Acp	crossbreeders	0.535	0.461	0.074	0.138
	inbreeders	0.000	0.000	0.000	0.000
Pgi	crossbreeders	0.676	0.606	0.070	0.104
	inbreeders	0.442	0.004	0.438	0.750
Pgm	crossbreeders	0.362	0.323	0.039	0.108
	inbreeders	0.000	0.000	0.000	0.000
Sdh	crossbreeders	0.322	0.288	0.034	0.106
	inbreeders	0.000	0.000	0.000	0.000
Pgd	crossbreeders	0.027	0.026	0.001	0.037
	inbreeders	0.000	0.000	0.000	0.000
Total	crossbreeders	0.384	0.341	0.043	0.099
	inbreeders	0.442	0.004	0.438	0.750

H_T = mean total allelic diversity, H_S = mean within population diversity, D_{ST} = mean between population diversity, G_{ST} represents the proportion of diversity distributed between populations. Only variable species and loci have been used for the calculation of the parameters.

the first Principal Component the populations are mainly separated on basis of the frequencies of the Acp-b, Acp-a, Pgi-b, Pgi-c and Pgm-a alleles. The left side of the scatterplot pictures the populations that contained 100% Acp-b alleles, populations in the right side of the scatterplot contained higher frequencies of the Acp-a allele. The populations in the left side also contained more Pgi-c alleles, whereas in the populations towards the right side of the scatterplot more Pgi-b alleles appeared. Populations in the left side of the scatterplot contained 100% Pgm-a alleles, this frequency of Pgm-a decreased towards the right side of the scatterplot. The most important variable on the second Principal Component was the Pgi-d allele. The populations in upper half of the scatterplot contained high levels of Pgi-d alleles, this allele was hardly found in populations in the lower half of the scatterplot. An other difference between the upper half and the lower half of the scatterplot was that the populations in the upper half contained less Pgi-b and Sdh-b alleles than the lower half. The scores of all

Table 6: Nei's Genetic Identity (Nei, 1972) summarized for seven *Lolium* species.

	1	2	3	4	5	6	7
1 *perenne*	.873						
2 *multiflorum*	.825	.946					
3 *rigidum*	.867	.915	.944				
4 *loliaceum*	.756	.883	.906	1.000			
5 *persicum*	.730	.817	.831	.850	.883		
6 *temulentum*	.754	.804	.814	.800	.850	.800	
7 *remotum*	.822	.853	.893	.801	.802	.802	1.000

populations on the first and third Principal Component are plotted in Figure 2. In this scatterplot the third Principal Component mainly separates the populations on basis of higher frequencies of Pgi-c and Sdh-c alleles and a lower frequency of Sdh-b in the lower half of the scatterplot.

Discussion

Examination of twenty-five populations of several *Lolium* species indicates that there is substantial allelic variation found within and between populations. Almost all populations are in Hardy-Weinberg equilibrium for al five isozymes studied. Only two populations, *L. multiflorum* (Landrace) for 6-Pgd and *L. rigidum* (Jabuk) for Acp, showed significant ($P \leq 0.01$) deviations from the Hardy-Weinberg equilibrium for one isozyme each.

Inbreeding species versus cross-breeding species

The proportion of variation distributed within- and between-populations depends on the breeding system of the population, showing very large differences between cross- and inbreeders, but small differences within these groups. In this study the inbreeding populations can be separated from the cross-breeding populations based on the almost complete lack of intra-populational variation compared to the cross-breeding populations (Table 4 and 5). Separation on basis of PCA is also well possible. The first Principal Component separates all inbreeding populations from the cross-breeding populations. This separation is mainly caused by the fixation for the Acp-b allele and the Pgm-a allele of the inbreeding populations. The cross-breeding populations contain next to these alleles also substantial amounts of the Acp-a (and to a lesser extent Acp-c, Acp-d and Acp-x alleles) and the Pgm-b allele (and to a lesser extent the Pgm-c and Pgm-d alleles, Table 3).

Inbreeding species

Very low electrophoretic variation is found for the populations of the four inbreeding species: *L. loliaceum*, *L. persicum*, *L. temulentum* and *L. remotum*. The parameter A is in all cases 1.00, except in one (A=1.2, Table 4). All populations are completely fixed. Hamrick & Godt (1990) report a value for A of 1.31 and a mean number of polymorphic loci (P) of 20% for inbreeding species. As the selfing populations in this study do not show within-population variation P is equal to 0%. In inbreeding species almost all variation is distributed between populations (Table 5).

Table 7: Percentage variation explained and the variables with the highest Principal Component loadings on the first three Principal Components.

PC	1		2		3	
% variation explained	32.02		20.92		14.04	
PC loadings	ACP-b	-0.47	PGI-d	0.85	PGI-c	-0.79
	PGI-b	0.36	PGI-c	-0.30	SDH-b	0.36
	PGI-c	-0.36	PGI-b	-0.28	SDH-c	-0.34
	ACP-a	0.35	SDH-b	-0.16	PGI-b	0.25
	PGM-a	-0.32	PGI-a	-0.14	PGI-c*	0.18

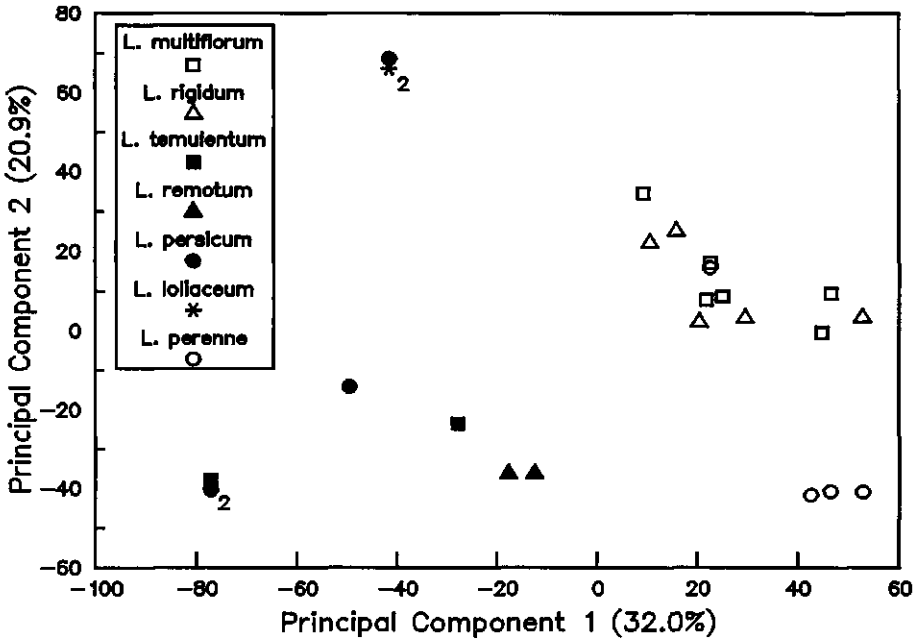

Figure 1: Scatterplot of 25 *Lolium* populations on the first two principal components, based on allelic frequencies. 2 two overlapping populations from the same species

Hamrick & Godt (1990) report that autogamous species have a mean H_T of 0.334 of which 51 % is distributed between populations (G_{ST}). These figures are based on the analysis of the data of 78 studies, with a mean number of 20.3 populations and 16.2 loci screened per study. In the present study the combined H_T for the inbreeding species (weighted for number of populations) gives a value of 0.442 of which 75% is distributed between populations, based on a mean number of 1 polymorphic locus (Pgi-2) and average 2.7 populations per species (Tab. 5). These figures indicate that the Pgi-2 locus is a highly variable locus, for which a large percentage of variation is distributed between the populations of the inbreeding *Lolium* species. Remarkable is that in all selfing populations the same alleles are found for four of the five isozymes screened (Table 3). This leads to the conclusion that in the PCA (Fig. 1) the populations of the inbreeding species are separated on basis of the allelic variant they contain at the Pgi locus. As all allelic variants found in the inbreeding species are also very commonly found in the cross-breeding populations, it is an indication that all *Lolium* species are extremely closely related.

Crossbreeding species

The mean number of alleles per locus is very high for the cross-breeding populations in this study. Hamrick & Godt (1990) report a value for A of 1.79. The value for P they report (49.7%) is also much lower than the value found in this study (P=80% or more). The high values for A and P in the present

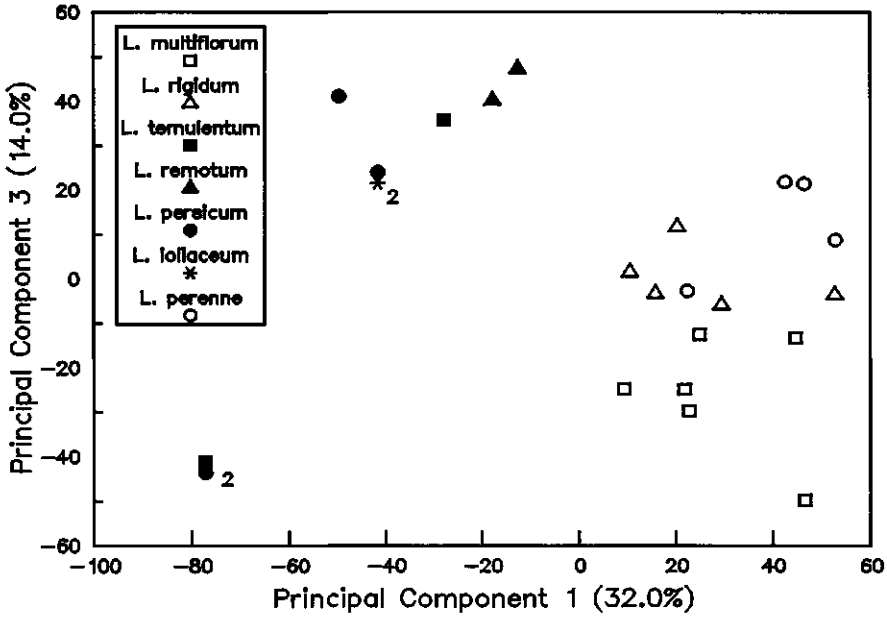

Figure 2: Scatterplot of 25 *Lolium* populations on the first and third principal component, based on allelic frequencies. 2 two overlapping populations from the same species

study can be explained by the limited number of isozymes used and the fact that all loci scored are polymorphic.

The distribution of the allelic variation of the cross-breeding species is very different from that of the inbreeding species. The figures given for wind-pollinating cross-breeding species by Hamrick & Godt (1990) are H_T =0.293 and G_{ST}=0.099 (based upon 134 studies, mean number of 10.7 populations and 16.7 loci screened per study), in this study a mean H_T of 0.384 and a mean G_{ST} of 0.099 are found (Tab. 5). These figures are based on a mean number of 4.7 loci and 5 populations analysed for each species. The level of variation in cross-breeding *Lolium* species is very high, and the part of the variation distributed within populations is large.

The *L. perenne* populations, except one, can be separated from the other two cross-breeding species on the second Principal Component (Figure 1). The almost complete lack of Pgi-d alleles and the almost complete fixation for the Sdh-b allele in the *L. perenne* populations causes this separation (Table 3 and 7). The PCA (Figure 2) also shows that *L. multiflorum* and *L. rigidum* can be separated on the third Principal Component. The higher frequency of Pgi-c and Sdh-c alleles and the lower frequency of the Sdh-b allele in the *L. multiflorum* populations causes this separation (Table 3 and 7). The genetic identity (Table 6) between these two species is the highest found between any two *Lolium* species. This agrees with the findings of Bulinska-Radomska & Lester (1985) who reports that on the basis of

seed proteïn similarity *L. multiflorum* and *L. rigidum* are most alike.

Morphological versus isozyme variation

During an earlier study on the morphological variation in *Lolium* populations (Loos, in press) and in this study on the isozymic variation in *Lolium* populations, largely the same populations have been used. It seems valid to compare the results of both kinds of analysis, to evaluate the use of both methods for measuring genetic variation and determination of species distinction.

When the results for individual populations are compared, two striking facts are obvious. The *L. perenne* population originating from Turkey, appears to be both on morphological and isozyme data more like a *L. rigidum* population. So it can be concluded that it is in fact a *L. rigidum* population. The *L. multiflorum* population originating from Cyprus looks on basis of morphological data more like a *L. perenne* population. But the isozymic analysis classifies it as a *L. multiflorum* population. This could indicate that genes have been exchanged between *L. perenne* and *L. multiflorum* in the past, of which traces still can be found.

Both morphological and isozyme analysis clearly separate the inbreeding populations from the cross-breeding populations. But whereas morphological analysis distinguishes, within the inbreeding species, *L. remotum*, *L. loliaceum*, and *L. persicum* combined with *L. temulentum*, isozyme analysis only separates *L. loliaceum* from the rest of the inbreeding species. But even these *L. loliaceum* populations are identical to one *L. persicum* population (Table 3, Figure 1). This indicates that species distinction within the inbreeding group based on isozyme data is impossible in this experiment. This could be caused by the small number of isozymes scored. Within the cross-breeding group, morphological data indicate that the three species can be distinguished, but these three species are very close related. The isozyme data show that the separation of *L. perenne* populations from *L. multiflorum* and *L. rigidum* is well possible. The separation of the latter two species is also possible. Both on morphological and isozyme data it can be concluded that the three cross-breeding species are close but can be distinguished. Based on both the morphological and the isozymic data *L. loliaceum* seems to be closely related to *L. rigidum*. This agrees with the view-point that *L. loliaceum* is more related to the cross-breeding species than any other inbreeding species (Thomas, 1981), and the fact that the close relationship between *L. loliaceum* and *L. rigidum* is sometimes stressed (Terrell, 1968). *L. remotum* appears to be intermediate between the cross-breeding and the inbreeding species based on both kinds of studies (Table 6, Figure 1).

Concluding, morphological analysis separates the inbreeding *Lolium* species better than isozyme analysis. This seems obvious as species have been distinguished on morphological differences. For the cross-breeding species morphological and isozyme analysis are equally successful in separation of the species, but it should be reminded that the isozyme analysis is bases on a limited number of populations. As all cross-breeding *Lolium* species originally have different distribution areas (Terrell, 1968), shifts in allelic variants and frequencies can also be associated with geographical distribution. In order to determi-

ne if this association exists a more extensive study into the genetic variation within *L. perenne* across its distribution area in Europe is carried out.

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CHAPTER 4:

SEED CHARACTERS IN *LOLIUM*, MORPHOLOGY AND PROTEIN CONTENT

B.P. Loos and J. Piket

Introduction

Seed characters have been described for several *Lolium* species. Terrell (1968) used only one seed character in his determination key to mature plants, to distinguish two groups in the genus. *Lolium temulentum* L. and *L. remotum* Schrank are described as having plump and thick caryopses, only 2-3 times longer than wide; *L. rigidum* Gaud., *L. persicum* Bois. & Hohen. and *L. canariense* Steud. are described as having caryopses more than three times longer than wide. In Table 1 more information is given on the dimensions and colour of the caryopses.

Little other information is available on morphological seed characters of *Lolium*. Only for the species *L. remotum* and *L. temulentum* a more detailed description is available, as they are known as weeds in flax and cereals. Musil (1963) gives a key to the seed characters of several *Lolium* species. Characters of the awns are not considered: he remarks that awns often break off and do not form a reliable character. As main distinction between *L. perenne* L., *L. multiflorum* Lam. and *L. persicum* on one side and *L. remotum* and *L. temulentum* on the other side, Musil gives the oval seed shape and strongly arched ventral side in the lateral view of the latter. *L. perenne*, *L. multiflorum* and *L. rigidum* are not distinguishable according to Musil (1963)

Table 1: Seed dimensions of 7 *Lolium* species

Species	Length caryopsis (mm)	Width caryopsis (mm)	Colour
L. perenne	2.9 - 5.5	0.7 - 1.5	light brown*
L. multiflorum	2.6 - 3.8	0.7 - 1.5	light brown*
L. rigidum	2.7 - 5.5	1.0 - 1.4	brown to blackish
L. canariense	3.3 - 4.5	0.8 - 1.2	light brown*
L. temulentum	4.2 - 7.0	1.6 - 3.0	light-dark brown
L. remotum	3.2 - 4.5	1.2 - 1.8	brown
L. persicum	5.5 - 7.0	1.2 - 1.9	light-dark brown

All information from Terrell (1968), * own observations

L. remotum, *L. temulentum* and *L. persicum* can be distinguished on basis of differences in seed shape, seed length and location of the broadest point (Musil, 1963).

A problem that arises in determining seed dimensions is the need to measure a representative sample of seeds. As the seeds are generally not very large, this is a

Table 2: Populations used for seed character determinations

no.	species	accession	origin/cultivar
1	*L. perenne*	CGN09985	Netherlands
2		15879-74	Turkey
3		864776	Czechoslovakia
4		864799	Czechoslovakia
5		15847-74	Turkey
6		6-327	Austria
7		491-1-76	Germany
8	*L. hybridum*	S81.913	'Grassland Manawa'
9		S81.901	'Pilot'
10		S81.914	'Grassland Ariki'
11	*L. multiflorum*	90.912	USA
12		90.913	USA, unawned
13		90.943	USA
14		90.944	USA
15		90.946	USA
16		82.035	'Landras'
17		82.059	'Vitesse'
18		82.908	'Rocket'
19		83.946	'Menichetti'
20	*L. rigidum*	90.905	'Wimmera'
21		90.906	selection Wimmera
22		90.914	south Australia
23		90.915	Victoria
24		90.916	Jabuk
25		90.917	Reeves Plain
26		90.919	Crete
27		90.938	Evia
28		CGN12511	Piemonte
29		90.942	Japan
30	*L. remotum*	GRA 426/83	-
31		GRA 418/81	-
32	*L. temulentum*	90.920	Lazio
33		86.4885	Pakistan
34		86.4886	Pakistan
35	*L. loliaceum*	90.937	Peloponnesis
36		90.939	north Crete
37		90.940	-
38	*L. multiflorum*	PI 277848	Cyprus
39	*L.persicum*	PI 239727	Iran
40		PI 239729	Iran
41		PI 239728	Iran
42		PI 230110	Iran
43		PI 229764	Iran
44		PI 222807	Iran
45		PI 269386	Afghanistan
46		PI 317450	Afghanistan
47		PI 163283	India
48		PI 314446	ex-USSR
49		90.941	-
50	*L. canariense*	INRA	Canary Islands

burdensome and time consuming activity. Image analysis can be helpful in raising the accuracy level of the measurements and reducing the time needed to measure one sample (van Vooren et al., 1991).

Next to morphological seed characters also biochemical characters of the seeds can be considered. Total protein electrophoretic pattern is one of these biochemical characteristics. Bulinska-Radomska & Lester (1988) and Gardiner & Forde (1992) describe electrophoresis protocols to determine banding patterns for total protein content in *Lolium*. This character is mainly used to characterize the numerous *L. perenne* cultivars, and appears to be a useful tool in discrimination. About the intra-specific and interspecific variation in the protein banding patterns little is known.

The objective of this study is to evaluate image analysis for the measurement of metric seed characters, and to determine the discriminating power of the characters measured. Also the discriminating power of protein banding patterns is evaluated.

Material and methods

Photographs of the seeds of eight *Lolium* species are given in Figure 1. The used *Lolium* populations are tabulated in Table

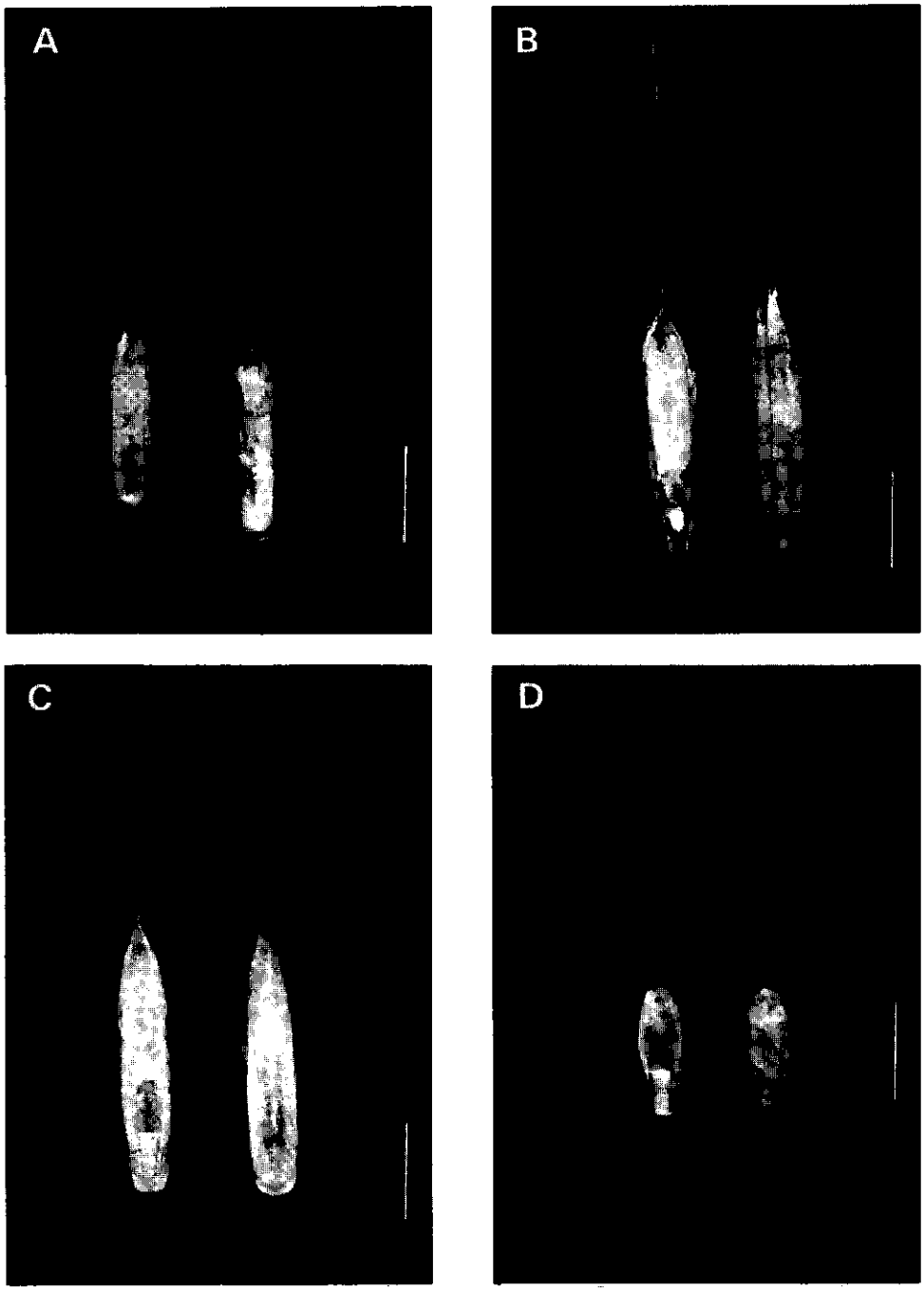

Figure 1: Photographs of the ventral (left) and dorsal (right) side of the seeds of eight *Lolium* species. A *L. perenne*, B *L. multiflorum*, C *L. rigidum*, D *L. loliaceum*. The bar represents 3 mm.

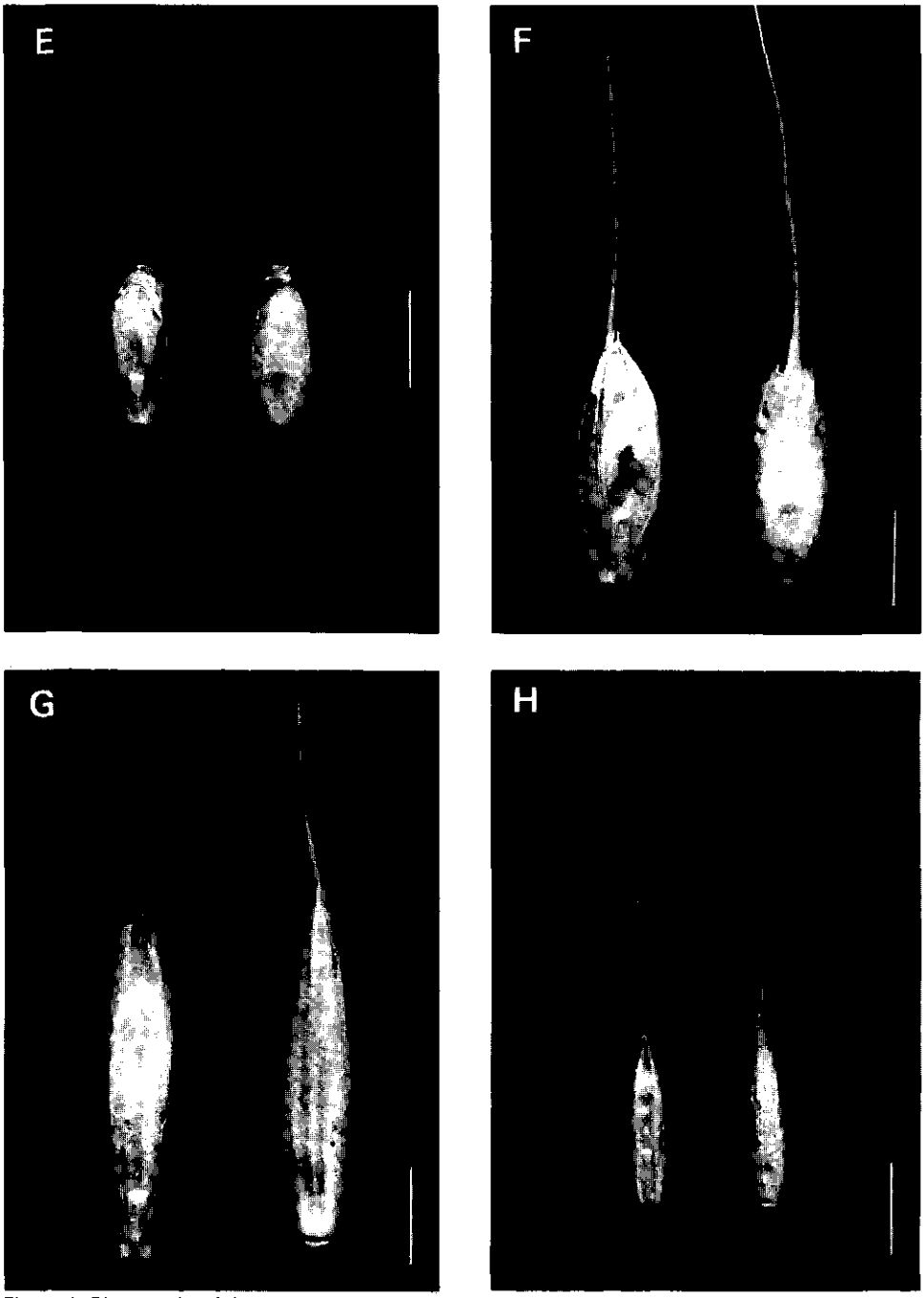

Figure 1: Photographs of the ventral (left) and dorsal (right) side of the seeds of eight *Lolium* species. E *L. remotum*, F *L. temulentum*, G *L. persicum*, H *L. canariense*. The bar represents 3 mm.

2. Number, species and origin or cultivar name (if possible) are given for each population. Most of these populations were also used in the studies on plant morphology and isozyme variation (chapter 2 and 3, this thesis). One species name is added to the set of populations: *Lolium hybridum* Hausskn., this is the hybrid between *L. perenne* and *L. multiflorum*.

Image analysis

From each population sixty seeds were measured using image analysis. The used technical equipment was a CCD camera (Sony XC 77CE) with a 20-100 mm zoomlens, an Apple II ci computer with 8Mb internal memory, a data translation frame grabber DT 2255 and the software programme Scil_Image version 1.2. Seeds were placed on a light box, not touching each other. The image was recorded and translated into an image consisting of 400 by 400 pixels. Several characters were measured in this binary image:

- length of the smallest enclosing rectangle (LR)
- width of the smallest enclosing rectangle (WR)
- area (A)
- perimeter (P)
- shape measure ($S = P^2/(4\pi \times A)$)
- ratio length-width (R= LR/WR)

The awns carried by some seeds had to be omitted from the measurements, as they would disturb the comparison of samples. From the binary image of each seed two layers of pixels were peeled of, which removed all the awns. Hereafter two layers of pixels were added to the outline of each image, restoring the original outline of the seeds, now without awns. All measurements were taken on the ventral or dorsal side of the seeds as this was the natural position of all seeds. It was not possible to measure the seeds from the lateral side. Next to these characters the weight of 100 seeds (W) was taken for each population as an additional character.

Electrophoresis

All recipes used for SDS polyacryl amide electrophoresis (SDS-PAGE) were according to Gardiner & Forde (1992). For each population 200 seeds were crushed using a bullet shaker during 5 minutes at 1800 movements a minute. Forty mg of seed powder was weighted out and transferred to an eppendorf cup, half a ml of extraction buffer was added. The samples were mixed on a vortex and left overnight at room temperature. The next morning samples were again mixed on a vortex, and placed in a waterbath of 85 °C for ten minutes. A slit was made in the cap to avoid build up of pressure. Hereafter the samples were cooled to room temperature and centrifuged for ten minutes at 1000 rpm. Electrophoresis was performed with a running gel of 11% polyacryl amide and a stacking of 5% polyacryl amide. The run was performed according to the conditions described by Gardiner & Forde (1992), only the amperage and wattage were adapted for the longer and wider (22 cm * 20 cm * 1,5 mm) gels used in this study. The power supply was kept at 110 mA for one hour, then turned to 64 Watt for four hours. The populations *L. perenne* 'Barenza' and *L. persicum* (PI239729) were represented twice on each gel as band

Table 3: Correlations between the 7 observed characters

	LR	WR	A	P	S	R	W
LR	1.000						
WR	0.810	1.000					
A	0.979	0.902	1.000				
P	0.999	0.833	0.986	1.000			
S	0.810	0.323	0.679	0.785	1.000		
R	0.766	0.254	0.624	0.738	0.996	1.000	
W	0.775	0.917	0.852	0.791	0.359	0.295	1.000

markers. After the total protocol described by Gardiner & Forde (1992) was performed, an additional staining was done according to Blakesley & Boezi (1977), to enhance colouring of faint bands. The gels were scored for absence (0) or presence (1) of bands. In total 48 bands could be observed resulting in a data matrix consisting of 50 populations with 48 variables.

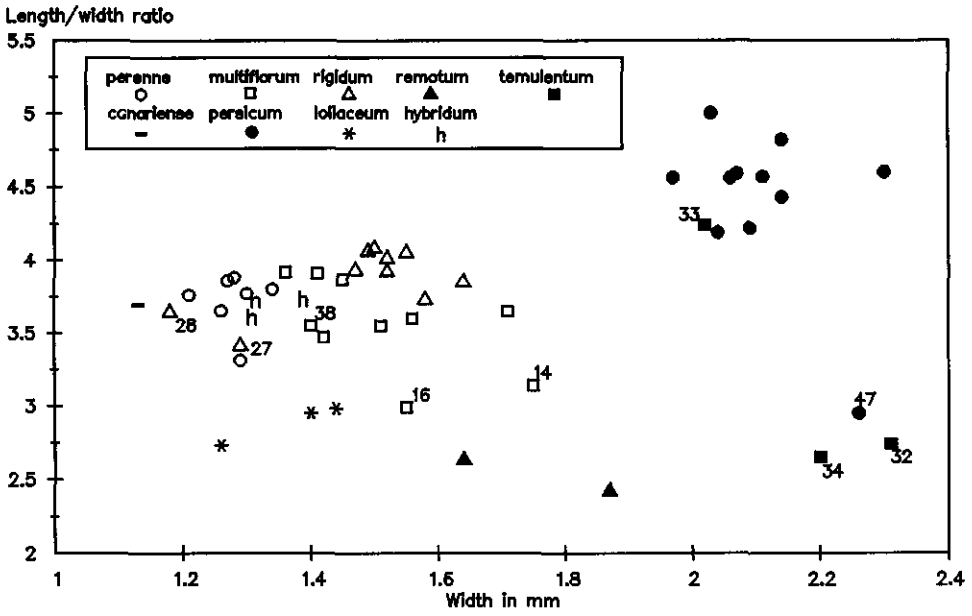

Figure 2: Scatterplot of fifty *Lolium* populations, using mean width and length/width ratio along the axis population numbers are according to Table 2 and are used in the text

Data analysis

As all characters from the image analysis were measured in pixels, they had to be transformed into mm and mm^2 using a calibration factor in mm (400 pixels = 49 mm, 160 000 pixels = 2401 mm^2). Means and standard deviations were calculated for all morphological characters measured.

Duncans multiple range test (DMRT, $P \leq 0.01$) was used to test for significant differences between the *Lolium* species, for this test all populations from each species were grouped. Linear correlations between the characters were calculated.

From the binary data matrix containing the electrophoresis results, Jaccard's similarity (1) measure was calculated between each population.

Jaccard similarity coefficient

$$J = M/(T1 + T2) - M \qquad (1)$$

M = number of bands in common between two samples, T1 = total number of bands observed in sample 1, T2 = total number of bands observed in sample 2.

The similarity matrix was used for clustering of the populations, using the unweighed pair group method average (UPGMA) algorithm. The resulting dendrogram was used to find nine groups, agreeing with the number of species entered in the analysis.

Results

The correlations between the 6 characters measured with image analysis plus the 100 seed weight are given in Table 3. The general level of correlation was high. Only width of the seed and seed shape (WR - S), width of the seed and ratio length/width (WR - R), shape of the seed and weight (S - W) and ratio of the length and width and weight (R - W) had lower correlations. Due to the high correlations most characters had the same discriminating power. Plotting of the two least correlated characters (which have high correlations with all other characters measured) in a scatterplot represented the separation of all populations fairly well. This bi-plot is represented in Figure 2, using width of the seeds and length/width ratio on the axes.

Based on Figure 2 it was concluded that population 47 (PI163283) should be classified as a *L. temulentum* population and population 33 (86 4885) as a *L. persicum* population. The mean and standard deviation for each species were determined, grouping the populations, results are given in Table 4. With DMRT significant differences between species were calculated, based on grouping of the populations according to species label. Results are given in Table 5. All species are significantly different from each other in more than one character. Width of the seeds was the character which separated all species best. *L. perenne* and *L. hybridum* are most alike, they only differ in width of the seeds. The seed shape (S) of *L. hybridum* is not distinct from the seed shape of *L. multiflorum*. The seed shape (S) and the length/width ratio (R) of the seeds of *L. canariense* is similar to the seeds of three cross-breeding species, *L. perenne*, *L. multiflorum* and *L. hybridum*. The seeds of *L. persicum* and *L. rigidum* are significantly different at all characters from all other *Lolium* species. Although seeds from *L. temulentum*

Table 4: Mean and standard deviation for 6 seed characters of nine *Lolium* species

Species		LR	WR	A	P	S	R
perenne	M	4.74	1.28	4.64	10.29	1.85	3.72
	SD	.80	.16	1.24	1.64	.17	.47
hybridum	M	4.87	1.33	4.95	10.63	1.83	3.70
	SD	.63	.14	.99	1.32	.17	.45
multiflorum	M	5.34	1.51	6.13	11.64	1.79	3.56
	SD	.76	.21	1.51	1.57	.19	.50
rigidum	M	5.69	1.47	6.50	12.36	1.91	3.87
	SD	1.00	.21	1.88	2.10	.18	.46
loliaceum	M	3.93	1.37	4.11	8.80	1.52	2.89
	SD	.63	.17	1.05	1.32	.13	.36
remotum	M	4.38	1.75	5.86	10.09	1.40	2.52
	SD	.41	.23	1.11	.92	.09	.26
temulentum	M	6.26	2.26	10.35	14.00	1.52	2.78
	SD	.72	.23	1.83	1.45	.11	.29
persicum	M	9.43	2.09	14.78	20.16	2.21	4.53
	SD	1.13	.21	2.90	2.32	.18	.44
canariense	M	4.12	1.13	3.55	8.98	1.83	3.69
	SD	.58	.15	.71	1.18	.18	.55

M mean, SD standard deviation

are larger than *L. loliaceum* seeds, both species do not differ in seed shape (S) and length/width ratio (R).

Of the 50 populations screened with SDS electrophoresis only three pairs had identical banding patterns: the two *L. remotum* populations, *L. persicum* population 39 and 40, and two *L. rigidum* populations 'Wimmera' and a selection from 'Wimmera' (no 20 & 21, Table 2).

The results from the cluster analysis based on the Jaccard's similarity coefficients is plotted in Figure 3. At the nine group level all populations from *L. loliaceum*, *L. remotum*, and *L. canariense*, are grouped in a separate cluster. Two *L. rigidum* populations are classified separately from the rest of the populations from this species. One population (no. 24, Table 2) is classified in it's own cluster, the other population (no. 28, Table 2 is grouped together with all *L. perenne* populations, one *L. multiflorum* (no 38, Table 2) population and one *L. hybridum* population. The other populations from *L. hybridum* are grouped in a cluster containing most *L. multiflorum* populations. One *L. persicum* population (no. 47, Table 2) forms a separate cluster, all other *L. persicum* populations were in the same cluster as all *L. temulentum* populations.

Discussion

Seed characters have been scarcely used as determination character in *Lolium* species. Results from this study indicate that seed characters can be used as determination characters. Although on average (Table 4) all species are significantly different, Figure 2 illustrates that individual populations of the cross-breeding species show some overlap. Some problems arise with the seeds of *L. hybridum*, the hybrid between *L. perenne* and *L. multiflorum*. The seeds from the cultivars with this name tends to be similar to one of the parents, the same holds for the seed

protein pattern. This does not necessarily have to be the same parent for both types of characters. The combination of morphological seed characters and seed protein banding patterns can well be used for detecting hybrids within the genus *Lolium*. Condition is that the morphological seed characters are measured using image analysis, as it is otherwise impossible to measure all characters on a large sample with so much accuracy.

Table 5: Characters significantly different with DMRT ($P \leq 0.01$), for 9 *Lolium* species

Species	characters	Species	characters
perenne hybridum	WR	hybridum multiflorum	LR, WR, A, P, R
perenne canariense	LR, WR, A, P	hybridum remotum	LR, WR, A, S, R
hybridum canariense	LR, WR A, P	hybridum loliaceum	LR, A, P, S, R
multiflorum canariense	LR, WR A, P	multiflorum remotum	LR, WR, P, S, R
loliaceum canariense	WR, A, S, R	remotum canariense	WR, A, P, S, R
temulentum loliaceum	LR, WR, A, P	perenne remotum	LR, WR, A, S, R

All other combinations of species are significantly different for all characters

The Jaccard's similarity coefficients observed in this study are low, in the dendrogram nine groups are found before the similarity reaches 0.40. Bulinska-Radomska & Lester (1985, 1988) report higher Jaccard's similarity coefficients within the genus *Lolium* (≥ 0.51) and between *Lolium* populations and populations from the genera *Vulpia* and *Festuca* (≥ 0.35). This could be caused by the fact that Bulinska-Radomska did not use SDS-polyacrylamide electrophoresis, but high pH standard electrophoresis to detect the banding pattern. In both the present study and the studies of Bulinska-Radomska & Lester (1985, 1988), the number of bands detected in a sample varied from about 10 till 20. In total 21 bands could be detected using high pH electrophoresis within the genus *Lolium*. SDS electrophoresis revealed 48 bands. The larger number of populations and species used in the present study (50 populations of 9 species versus 14 populations of 5 species) could have resulted in the increase of bands found. Obviously many bands were only detected in some or single populations, therefore decreasing M. As T1 and T2 were at the same level in both studies, the similarity between two samples was reduced.

As most populations used in the present study were also analyzed using morphological characters of the whole plant (Chapter 2) and allozyme analysis (Chapter 3), these results can be compared. For most populations results from all three studies agree well, but for some they don't. The largest problems arise between populations of *L. persicum* and *L. temulentum*. Based on plant morphology the populations of both these species are divided into two groups: 1) *L. temulentum* population from Lazio (no. 32, Table 2) formed a group with two *L. persicum* populations (no. 47 and 49, Table 2)

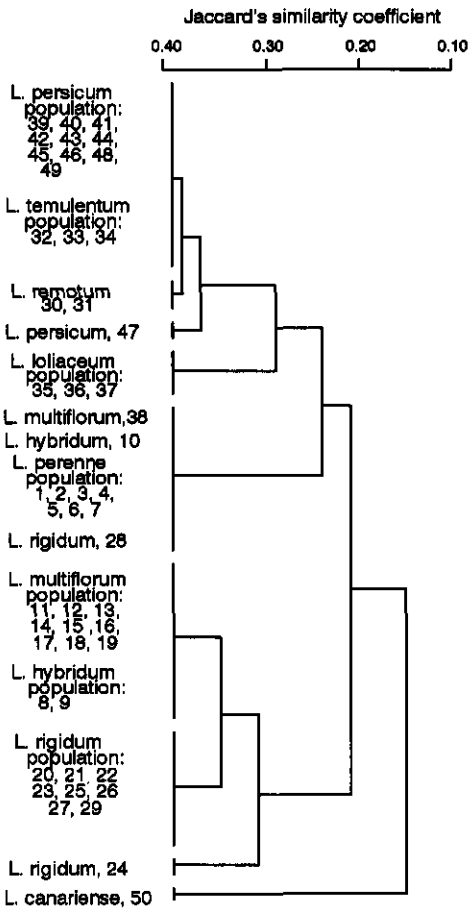

Figure 3: dendrogram of 50 *Lolium* populations, using Jaccard's similarity coefficient and the UPGMA algorithm

and 2) *L. temulentum* populations no 33 and 34 (Table 2) formed a group with all rest of the *L. persicum* populations. With the allozyme variation observed both species could not be separated as they were fixated for the same alleles at most loci studied. Based on morphological seed characters an other pattern appears. *L. temulentum* population from Pakistan (no 33, Table 2) is more similar to *L. persicum*, and *L. persicum* population from India (no 47, Table 2) is more similar to *L. temulentum*. The seed protein banding pattern only reveals *L. persicum* population from India (no 47, Table 2) as different (Fig. 3). All other populations group according to the species. Summarizing, there seems to be an conflict between morphological plant characters, seed characters and the protein banding pattern of the populations of *L. temulentum* and *L. persicum*. On two sets of data both species show large similarity, plant characters and protein pattern. Only for seed characters both species are somewhat more different. This could indicate that all screened populations are merely two forms of the same species, differing only in seed morphology. An explanation could be that both species are mimicry weeds in cereals (Dore, 1950). Depending upon among which cereal crop the *Lolium* population was growing, populations were selected for either plump seeds (*L. temulentum* like) or long, slender seeds (*L. persicum* like).

Based on morphological seed characters *L. rigidum* populations from Evia and Piemonte (no. 27 and 28, Table 2 and Fig. 2) appear like *L. perenne*. Population no. 27 appears, on basis of morphological plant characters, allozyme pattern and seed protein

pattern, as a *L. rigidum* population. The similarity found with *L. perenne* falls within the natural variation in seed characters of *L. rigidum*. Population no. 28 appears on seed protein pattern and seed morphology alike *L. perenne*. No morphological adult plant data and allozyme data are available for this population. Results indicate that the population could be misclassified, and should be named *L. perenne*.

For *L. multiflorum* populations 14 and 16 (Table 2) the seed morphology seems somewhat different (Figure 1). However based on plant morphology, allozyme patterns and seed protein spectra, both populations are *L. multiflorum* populations. The observed seed dimensions fall within the natural variation found within *L. multiflorum*. The *L. multiflorum* population from Cyprus (no 38, Table 2) was, based on plant morphology, more like a *L. perenne* population (Chapter 2). The allozyme pattern was similar to all other *L. multiflorum* populations, whereas the seed morphology could be either *L. perenne* or *L. multiflorum*. The seed protein banding pattern was like a *L. perenne* population, again suggesting that this population is a *L. perenne* population introgressed with *L. multiflorum*.

The single population of *L. canariense* has seeds similar to the cross-breeding species. The protein banding pattern is very distinct, indicating that *L. canariense* is a separate species. No plant morphology data or allozyme data were available for this population.

Concluding, seed protein banding patterns are very helpful in distinguishing between *Lolium* species, and also between the populations within these species. Metric seed characters were also valuable for species distinction, but care should be taken as the variation within species causes some overlap, which makes determination based only on these characters somewhat unreliable. Based on seed dimensions, population distinction within the species is often not possible.

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CHAPTER 5:

MORPHOLOGICAL VARIATION IN DUTCH PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.) POPULATIONS, IN RELATION TO ENVIRONMENTAL FACTORS

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Summary

Twenty-one Dutch *Lolium perenne* populations, fifteen European populations and six *L. perenne* cultivars were compared for morphological variation. Dutch populations clearly differed from the European populations and the cultivars. Dutch populations generally had reduced plant length and smaller leaves. For other characters, e.g. date of ear emergence, the Dutch populations showed as much variation as the European populations and cultivars they were compared with in this trial. Correlations between morphology and environmental factors at the site of origin were significant in several cases but were generally weak, and dependent on the set of populations studied. In order to maximize the phenotypic differences between the collected populations, soil type and management type appeared to be the most important factors for the choice of collection sites in the Netherlands. To determine suitable collection sites all over Europe, other factors like precipitation, latitude, altitude and temperature factors were also important. For subsampling of the locations, variation in management type within the location determined whether or not phenotypic different samples could be collected. The extensive use of cultivars in the Netherlands does not seem to have prevented the formation of distinct populations. Therefore *in situ* conservation of grassland seems a good alternative for genetic conservation of *L. perenne* in the Netherlands.

Introduction

Although *Lolium perenne* L. (perennial ryegrass) has been known for centuries as a fodder species, its agronomical importance increased significantly during the last century. In the Netherlands, about 150 000 ha of grassland is annually seeded or reseeded (Rassenlijst, 1993) on a total of about one million ha of grassland. The percentage of *L. perenne* seeds in the used seed mixtures has increased from about 45 % in the period of 1953-1965 to 84% in 1991/92. The tonnage of seed mixtures used also increased during this period.

The first breeding activities already took place in the 18th century in England by a.o. Pacey (Beddows, 1953). The first ryegrass cultivars appeared on the Dutch variety list in 1927, all seed stocks being derived from foreign material. In 1934 the first cultivars based on Dutch material were listed: 'Ceres' and 'Brabantia'. Extensive breeding programmes were started after 1940 in the Netherlands, at first concentrating on the

development of good fodder types (Vos, 1983). Later this century, cultivars were developed for more diverse purposes. This resulted in the separate listing of cultivars adapted for different applications, e.g. lawns and sportfields, since 1974 on the Dutch variety list.

Increasing economic importance also resulted in increased collecting of *L. perenne* populations. These collections were stored in genebanks or used in breeding programmes. These populations are of great importance to perennial ryegrass breeding. In the early days of perennial ryegrass breeding collected plants or seeds from several areas were combined, multiplied and exposed to mild selection pressure, to compose a new cultivar. Although breeding methods have become more advanced, it is assumed that breeding success still largely depends on the quality of the basic breeding material. In the past Dutch cultivars were largely obtained using collections of Dutch material, but during the last ten years the use of Dutch populations has decreased. Due to the widespread practice of (re)seeding and the intensive sampling of grassland in the Netherlands in the past, the assumption was made that no genetically new material could be collected in the Netherlands (Glas, 1983). Aim of the present study is to assess the genetic variation of Dutch populations and to compare these to European populations and cultivars to determine whether Dutch populations constitute a valuable part of the genetic resources of *L. perenne* in Europe.

The success of subsampling of the Dutch locations is determined. Tyler and Chorlton (1976) state that on very small areas large differences in phenotype can be found, suggesting that intensive sampling of variable locations can result in genotypically different samples.

Material and methods

Plant material

In October 1990 twenty-one *L. perenne* populations were sampled from seven locations in the Netherlands. Selection criteria for the locations were: the grassland had not been seeded or reseeded for at least fifteen years, the location had been managed either by grazing, haying or a combination of both, and the locations comprised an area of at least 30 ha. The locations were selected by using the information provided by Provincial Landscape Organisations, owning and managing the grassland. On each location three populations were collected consisting of 60 plants each. To avoid duplicate sampling, plants were collected at least 0.5 meters apart. The sites within each location were spaced about 100 - 300 meters. Each site was chosen to represent a contrast within the location (e.g. path versus wetland).

Besides the Dutch populations, fourteen populations from the European Forages database (Tyler, 1989) were selected and obtained through the Welsh Plant Breeding Station. These populations were selected on basis of their country of origin and their classification as semi-cultivated. Additionally one Austrian ecotype was obtained from Mr. Klein-Geltink (CPRO-DLO).

Besides the populations, six *L. perenne* cultivars were chosen, which had been on the Dutch variety list for over thirty years. These varieties have been used extensively over the years and most of them are based on Dutch populations, thus representing a part of the

Table 1: Populations and cultivars of *L. perenne* used in the trial (1991-1992)

No.		town	long.-lat.	number	origin	alt.	site physiology
1	NL	Peize	6.30E-53.10N	90.101	collected	1	Grassland near streamside with
2	NL	Peize		90.102	collected		variations in wetness and abundance
3	NL	Peize		90.103	collected		
4	NL	Yde	6.35E-53.07N	90.201	collected	6	Grassland near streamside with
5	NL	Yde		90.202	collected		variation in wetness, management
6	NL	Yde		90.203	collected		and abundance
7	NL	Balkbrug	6.17E-52.39N	90.301	collected	3	Grassland near streamside and with
8	NL	Balkbrug		90.302	collected		variation in management, abundance
9	NL	Balkbrug		90.303	collected		and fertilization
10	NL	Zwolle	6.07E-52.32N	90.401	collected	1	Foreland with variation in treading,
11	NL	Zwolle		90.402	collected		flooding, management and
12	NL	Zwolle		90.403	collected		abundance
13	NL	Geldermalsen	5.15E-51.57N	90.501	collected	2	Grassland with variation in hedge
14	NL	Geldermalsen		90.502	collected		and tree growth and
15	NL	Geldermalsen		90.503	collected		abundance
16	NL	Neerijnen	5.17E-51.49N	90.601	collected	5	Foreland with variation in
17	NL	Neerijnen		90.602	collected		abundance and management
18	NL	Neerijnen		90.603	collected		
19	NL	Yerseke	4.08E-51.28N	90.701	collected	-1	Saltish grassland with variation in
20	NL	Yerseke		90.702	collected		wetness and abundance
21	NL	Yerseke		90.703	collected		
22	AU	Hilgau	11.48E-47.51N	90.800	collected	850	Path true meadow on valley slope
23	B	Moinet	5.50E-50.02N	9066.72	WPBS	500	Grassland on plateau, threaded
24	F	Moutiers	4.10E-47.34N	9081.81	WPBS	240	Grassland in valley bottom
25	F	Mirecourt	6.08E-48.18N	9113.72	WPBS	289	Grassland near stream-side in plain
26	F	Mirecourt	6.08E-48.18N	9110.72	WPBS	287	Arable land
27	GBW	Overton	2.56W-52.58N	9995.80	WPBS	0	Grassland
28	GBW	Gogidan	3.55W-52.25N	9793.00	WPBS	70	Waste land in valley bottom
29	GBW	Betsw-y-wyth	3.48W-53.05N	10411.85	WPBS	239	Grassland on terrace
30	GBE	Chirkton	3.03W-52.56N	9957.82	WPBS	100	Grassland on valley slope
31	GBE	Irby	0.13W-53.09N	11137.89	WPBS	0	Grassland with hedgerows
32	CH	Wald	8.56E-47.17N	10286.83	WPBS	1200	Grassland on valley slope with path
33	CH	Turbenthal	8.51E-47.27N	10284.86	WPBS	720	Grassland on valley slope
34	CH	Ballens	6.27E-46.34N	9088.82	WPBS	707	Grassland on plateau
35	I	Poirino	7.50E-44.55N	8591.00	WPBS	270	Irrigated grassland in plain
36	ROU	Tirgu	27.00E-47.12N	9975.81	WPBS	150	Grassland near stream side in valley

No		Cultivar name	Breeder	Number	Year	Origin
37	NL	Perma	Cebeco	80.003	1939	Collection from Schouwen-Duiveland
38	NL	Lamora	Mommersteeg	80.005	1945	Collection from Western Brabant
39	NL	Barenza	Barenbrug	80.016	1949	Collection from Krimpenerwaard
40	B	Vigor	Rvp lemberge	80.023	1948	Collection from Flanders
41	NL	Barstella	Barenbrug	80.024	1955	Collection from Krimpenerwaard
42	DA	Hora	Cebeco	80.007	1939	Selection from 'Ceres', a danish cultivar

NL= The Netherlands, B=Belgium, F=France, DA= Denmark, GBW=Great Britain Wales, GBE=Great Britain England, CH=Switzerland, I=Italy, Rou=Romania. Long.=longitude, Lat.=latitude, Alt.=altitude WPBS=Welsh Plant Breeding Station. year= first year listed on Dutch variety list

Table 2: Characters measured on the collection of *L. perenne* populations and cultivars in 1991 and 1992

Char.	Description	year	axis1	axis2
DE	Date of ear emergence (days after the first of April)	91/92	0.91	-0.42
NH	Natural height at emergence (cm)	91/92	0.57	0.52
H	Habitus (1=erect to 9=prostrate)	92	0.24	-0.86
LF	Length of flag leaf (cm)	91	0.45	0.56
WF	Width of flag leaf (mm)	91	0.03	0.52
HA	Height 30 days after ear emergence (cm)	91/92	0.94	-0.06
LNE	Length of the upper internode and ear (cm)	91/92	0.91	-0.25
LN	Length of the upper internode (cm)	91/92	0.70	-0.20
EL	Ear length (cm)	91/92	0.93	-0.27
NS	Number of spikelets per spike	91/92	0.91	-0.05
LS	Length of the spikelet awn not included (mm)	91	-0.62	0.37
NF	Number of florets	91	-0.48	0.38
PA	Percentage awned florets	91	-	-
LA	Length longest awn (mm)	91	-	-

Char.= character, axis1 and axis 2= principal correlations of the characters with principal component number one and two.

variation of Dutch populations. Detailed information on all populations is given in Table 1.

Field design

The European populations and the cultivars were sown mid September 1990, and transplanted to the field at the end of October. The plants of the Dutch populations were planted in pots (18 by 18 cm) and kept outdoors during winter. In the beginning of March 1991 these plants were transplanted to the field. All plants were cut at 15 cm height. Each population consisted of 60 plants, layed out in the field in a complete randomized block design, with three blocks containing plots of twenty plants each. In the spring of 1991 the characters (Table 2) were measured on each plant. After the measurements all plants were cut back, this was repeated twice in 1991 and at the beginning of March 1992. In spring 1992, measurements were repeated (Table 2). One character was added: habitus, which was scored on March 25, 1992.

Data analysis

As the layout of the experimental field was identical in both years the heritability could be calculated for the characters observed in both years, using the average per plot for each character:

	Df	MS	E(MS)
Replication	r-1		
Year	y-1		
Genotype	g-1	MS_g	$\sigma_e^2 + ry\,\sigma_g^2 + r\,\sigma_{gy}^2$
Genotype × Year	(g-1)(y-1)	MS_{gy}	$\sigma_e^2 + r\,\sigma_{gy}^2$
Error	(r-1)(gy-1)	MS_e	σ_e^2

Df= degrees of freedom, MS= mean square, E(MS)= expected mean square

The heritability was calculated as:

$$h^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2/ry + \sigma_{gy}^2/y} \quad \text{(Nyquist, 1991)}$$

With g= number of populations (42), y=number of years (2), r= number of replications (blocks, 3), σ_e^2= expected residual variance, σ_g^2= expected genotypic variance, σ_{gy}^2= expected genotype×year variance.

The characters measured in both years (Table 2) were averaged for each plant to obtain estimations of population performance less affected by population×year interaction, these means were used during further analysis.

The characters were averaged and standardized per population before principal component analysis (PCA). The obtained principal component scores of the first six axes were used for clustering of the populations. These scores are uncorrelated characters. The Squared Euclidian Distance ($S(x,y)=\Sigma_i(x_i - y_i)^2$) was used as distance measure and Ward's method as clustering algorithm. The mean values of each population for each character were tested with the Least Significant Difference test (LSD, $P \leq 0.05$) for differences between clusters.

To assess whether the found morphological variation can be related to environmental factors, meteorological data for all locations of the Dutch and the European populations were obtained (Meteorological office, 1972; Wallen, 1970 and 1977; Rudloff, 1981). These data are generally based on observations during 30 years or more. Also some non-meteorological factors were determined for each location. All factors, that were used in the analysis, are tabulated in Table 3. As it was not possible to gather environmental factors for the cultivars, these were omitted from this part of the analysis. Correlations were calculated between the morphological characters and the environmental factors (except MAN, a qualitative factor), and between the scores of the populations on the first two principal components and the environmental factors (except MAN). The LSD test ($P \leq 0.05$) was used to find significant differences between the clusters for the environmental factors entered in the analysis (except MAN). For the factor grassland management four classes were distinguished: no management, only haying, only grazing, and haying with aftermath grazing. All classes were tested (LSD, $P \leq 0.05$) for significant differences of the morphological characters and the first two principal component scores.

To establish whether there were differences between subsamples within the Dutch locations, PCA was performed with the standardized data of each plant within each location. This was done for each location separately, seven analyses were performed in total. The scores of the individual plants on the first two principal components were plotted, to observe if there was grouping of the plants sampled on each sub-location.

Duncans multiple range test (DMRT, $P \leq 0.05$) was used to establish whether Dutch populations differed significantly at any character from each other. The number of significant differences were counted within (3 comparisons) and between (54 comparisons) locations and averaged. This average number of significantly different characters was considered as a dissimilarity measure within and between locations.

Table 3: Environmental factors and their abbreviation

Factor description	Abbre-viation	Range Min.	Max.
Number of months with a mean temperature > 10°C	Mean10	5	7
Number of months with a mean temperature > 20°C	Mean20	0	3
Number of months with a maximum daily temperature > 10°C	Max10	7	9
Number of months with a maximum daily temperature > 20°C	Max20	0	5
Minimum daily temperature of the coldest month	minCM	-8.3	2.2
Maximum daily temperature of the warmest month	maxWM	18.1	29.4
Mean daily temperature	MDT	6.3	12.0
Mean daily maximum temperature	MDT_{max}	11.1	16.7
Mean daily minimum temperature	MDT_{min}	1.5	7.8
Mean daily temperature range	MDT_{range}	5.3	10.3
Absolute maximum temperature	AB_{max}	27.0	39.6
Absolute minimum temperature	AB_{min}	-30.0	-5.0
Absolute temperature range	AB_{range}	32.0	69.6
Mean precipitation	MP	506	1388
Number of months with MDT < 0°C	MMDT	0	3
Number of months with MDT_{min} < 0°C	$MMDT_{min}$	0	8
Number of months with AB_{min} < 0°C	MAB_{min}	0	10
Altitude	ALT	-1	1200
Latitude	LAT	44.55	53.10
Grassland management	MAN	-	-

Results

Heritabilities

Date of ear emergence had the highest heritability (0.96), Number of spikelets per spike (0.93) had also a high heritability, the other five characters had lower heritabilities. The exact values being: Natural Height at ear emergence (0.89), Height 30 days after ear emergence (0.86), Length of the upper internode and ear (0.67), Length of the upper internode (0.68), Ear length (0.64).

Multivariate analysis

The characters Percentage awned flowers (PA) and Length of the longest awn (LA) were omitted from further analysis as too little variation was found for both these characters: the majority of populations had no awns. Twelve characters were entered in the principal component analysis. In Figure 1 the first two principal components are plotted, the first component explains 55.9% of the variation, the second 21.1%. The principal component correlations of the characters with the first two principal components (Table 2) showed that the populations orientated towards the right in Figure 1 are generally later (DE), larger after ear emergence (HA, LNE, EL) and have more spikelets (NS). On the second principal component (Fig. 1) the populations with the more prostrate growth habit (H), which are smaller at ear emergence (NH) and with smaller flag leaves (LF, WF), are located in the lower part of the Figure. The Dutch populations are clearly separated from most of

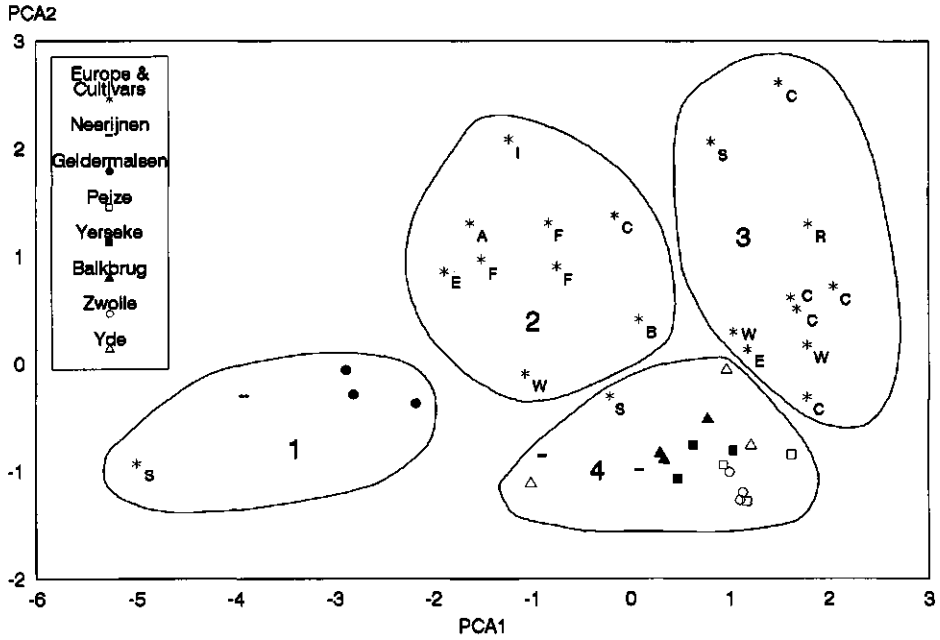

Figure 1: Scatterplot of 42 *L. perenne* populations on the first two principal components Europe= European populations, labelled with the following characters: A Austria, B Belgium, E England, F France, I Italy, R Romania, W Wales. Cultivars are labelled with C. The Dutch populations are represented by different markers, for location details see table 1. Number 1 to 4 indicate the used clusters.

the European populations and the cultivars by the second principal component. The dendrogram obtained after clustering of the populations showed four main clusters at the 75% between cluster variation level, these clusters are indicated in Figure 1. Cluster 1 contains four Dutch populations and one European ecotype from Switzerland. Cluster 2 contains eight European populations and one cultivar:'Barstella'. In cluster 3 all other cultivars are found and five European populations, cluster 4 contains the majority of the Dutch populations and one ecotype from Switzerland.

The results of the LSD-tests for significant differences between the clusters for both the morphological and environmental characters are in Table 4. Cluster 3 and 4 are most alike, they only differ at five of the twelve morphological characters used. All other combinations of clusters differ for, at least, nine characters. For the environmental characters the differences between the clusters are much smaller. Only Mean10, LAT, AB_{min} and AB_{range} showed significant differences between the clusters. Clusters separated only on the first principal component (cluster 1 versus 4 and cluster 2 versus 3) did no show any significant difference for environmental factors.

Table 4: Significant differences (LSD, $P \leq 0.05$) for morphological characters and environmental factors between each of four cluster groups (figure 1), for the abbreviations used, see table 2 and 3

Cluster 1 - 2	Morphologic	NH, H, LF, WF, HA, LNE, LN, EL, NS
	Environmental	Mean10, AB_{min}, AB_{range}
Cluster 1 - 3	Morphologic	DE, NH, LF, HA, LNE, LN, EL, NS, NF, LS
	Environmental	Mean10, AB_{min}, AB_{range}
Cluster 1 - 4	Morphologic	DE, NH, LF, HA, LNE, LN, EL, NS, NF, LS
	Environmental	none
Cluster 2 - 3	Morphologic	DE, NH, WF, HA, LNE, EL, NS, NF, LS
	Environmental	none
Cluster 2 - 4	Morphologic	DE, NH, H, WF, HA, LNE, LN, EL ,NS, NF, LS
	Environmental	Mean10, LAT, AB_{min}, AB_{range}
Cluster 3 - 4	Morphologic	NH, H, LF, HA, NS
	Environmental	Mean10, AB_{min}, AB_{range}

Environmental trends

The combinations of morphological characters and environmental factors which were significantly correlated are given in Table 5. These correlations indicate that later populations are found more to the north (LAT) and in less warm (MDT_{max}) climates. Taller plants at ear emergence can be found in climates where in more months a year the mean temperature is above 10° C (Mean10). A more prostrate growth habit points towards colder climates (maxWM, MDT_{max}) with lower temperatures during a longer period of the year (Mean10, Mean20, Max10, Max20) and with less temperature amplitude (MDT_{range}), a more northern distribution (LAT) and lower

Table 5: Significant correlations ($P \leq 0.01$) between morphological characters and environmental factors based on 36 *L. perenne* ecotypes

Morphological character	Environmental factors	Value
Date of ear emergence	LAT	0.47
	MDT_{max}	-0.44
Height at ear emergence	Mean10	0.45
Habitus	LAT, Mean10, Mean20,	0.78, -0.60, -0.54,
	Max10, Max20, maxWM,	-0.66, -0.43, -0.62,
	ALT, MDT_{max}, MDT_{range}	-0.46, -0.65, -0.45
Number of spikelets per spike	MMDT	0.44

altitudes (ALT). Fewer spikelets per spike are formed in climates where the mean daily temperature is below zero in more months a year (MMDT).

Calculation of the correlation coefficients between environmental factors and the first two principal component axes showed that none of the environmental factors used had a significant correlation with the first principal component. The second principal component was significantly ($P \leq 0.01$) correlated with several environmental factors: Mean10 (0.58), Mean20 (0.46), Max10 (0.45), maxWM (0.41), LAT (-0.67), and MDT_{max} (0.43).

The LSD test based on a grouping of the populations according to grassland management showed only little significant differences. One ecotype was eliminated in the analysis as no classification was given for management. For Number of spikelets a significant difference was found between populations from only hayed grasslands (18.7) and from only grazed grasslands (22.6). The value for populations from grasslands with a combination of both management forms was intermediate (21.5). The only other significant difference was found for Length of flag leaf, only grazed grassland (18.2) had larger flag leaves than grassland with a combined management (15.9). Hayed grassland had a smaller mean flag leaf length (15.2), but was not significantly different from the other two classes due to large variance.

Subsampling

Plots of the first two principal components of the plants collected at each location generally showed that plants from the three sublocations were scattered in one cloud of points. Only the populations collected at Neerijnen II (Figure 2a) and Yde II (Figure 2b) were separated from the other two populations collected at the same location.

DMRT showed several combinations of populations that did not differ significantly at any of the twelve characters. These combinations of populations were: Peize II & III, Balkbrug II & III, Zwolle I & III and Yerseke II & III. DMRT also showed that although populations within each location are very alike, they mostly differ on more than one character. It is possible that populations collected at different locations are identical: Peize II & III and Yde I did not differ at any character from Zwolle II. The mean number of characters on which populations differ within- and between locations are tabulated in Table 6. In general the within location Figures are lower than the between location Figures. Exceptions were the locations Yde and Neerijnen, which showed large within location differences. Peize, Zwolle and Yerseke showed little within location differences. The populations from Geldermalsen showed on average the largest differences with all other locations. The populations from Peize and Zwolle showed on average the smallest difference.

Discussion

Heritabilities

The estimated heritabilities indicate that the differences observed between the populations for these characters are largely genetically based. The heritabilities calculated for the seven characters observed in both years agree well with the literature. Elgersma (1990) reports heritabilities for some of the same

Figure 2A

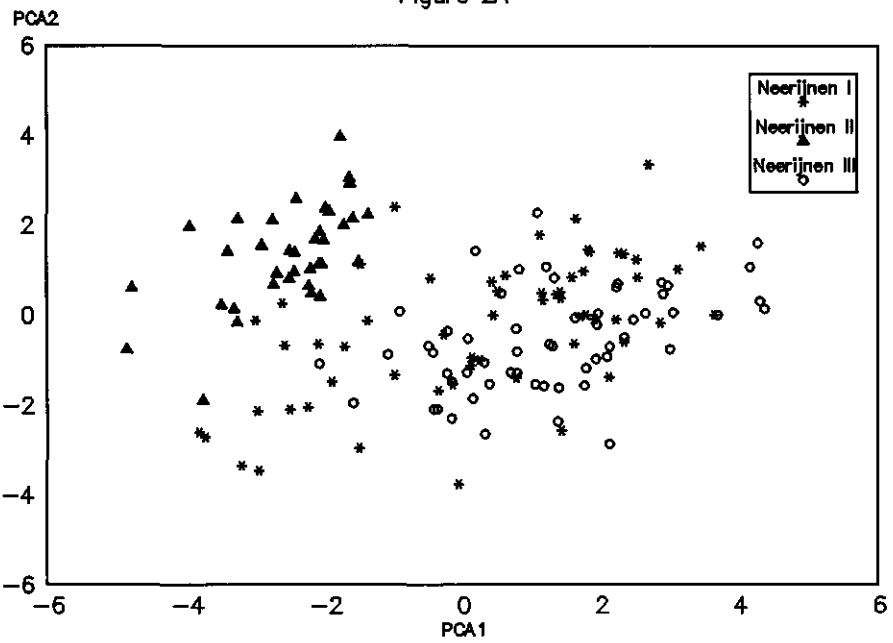

Figure 2B

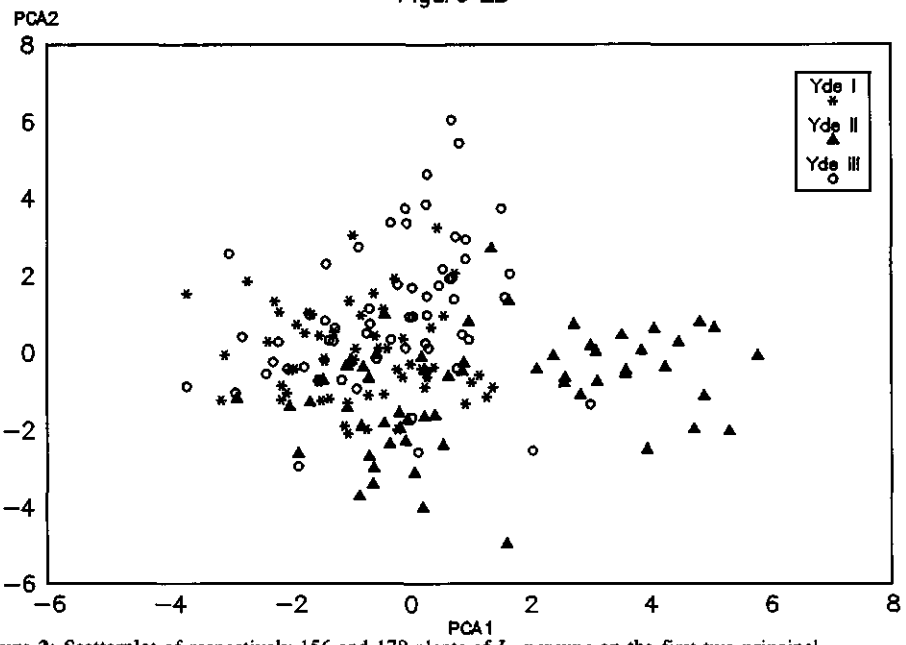

Figure 2: Scatterplot of respectively 156 and 178 plants of *L. perenne* on the first two principal components.

characters varying from 0.97 to 0.46, based on clones observed on two locations in one year. Date of ear emergence has the highest heritability. Humphreys (1991) reports a heritability for date of ear emergence of 0.98. This agrees with the present results that date of ear emergence has the highest heritability.

Variation in Dutch populations

All Dutch populations were clearly distinct from the Dutch cultivars. This may be caused by the fact that all cultivars used are medium/late heading fodder types. The Dutch populations in cluster 1 were clearly separated from the cultivars by their early heading date. The Dutch populations in cluster 4 were separated from the cultivars based on their more prostrate growth, smaller flag leaves and smaller height at ear emergence, but they are heading as late as the cultivars (Table 4). There is no indication that the Dutch populations are selections from the cultivars or that they are strongly influenced by introgression with cultivars.

The Dutch populations appear to be a specific group within *L. perenne*, compared to the European populations. They are distinct from most of the other European populations by their prostrate growth habit, smaller height at ear emergence and their smaller flag leaves (Fig. 1; axis 2). They do show large variation in date of ear emergence, height after ear emergence and number of spikelets (Fig. 1; axis 1). The sampling of the different locations within the Netherlands often resulted in the collection of different populations, although some locations resulted in very similar populations (Peize versus Zwolle). Especially sampling of the location Geldermalsen resulted in very specific forms of *L. perenne* (Fig. 1, Tab. 6). It is unlikely that this is caused by the selective force of meteorological factors, as the variation for these factors is not nearly as large in the Netherlands as in the whole of Europe. It is known that the location Geldermalsen is on an extremely heavy clay soil ('komklei') compared to the other locations, and this might have been an important selective force. One would expect that the location Yerseke should result in very specific forms of *L. perenne*, as this location is exposed to permanent salt stress. The populations from Yerseke are

Table 6: Average number of characters significantly different (DMRT, $P \leq 0.05$) within and between locations.

	P	Yd	B	Z	G	N	Ye
Peize	1.3						
Yde	5.1	7.3					
Balkbrug	5.3	5.9	2.7				
Zwolle	3.4	6.0	5.0	1.3			
Geldermalsen	10.0	9.0	10.2	10.1	4.0		
Neerijnen	8.1	7.9	7.8	7.9	8.9	8.0	
Yerseke	5.9	6.9	4.3	7.1	9.5	8.6	1.3

Maximum is 12.0.

however similar to populations from more northern, inland locations (Fig. 1, Tab. 6). Salt stress was not a selective force influencing the morphology of these *L. perenne* populations.

Division by clusters

The clusters (Fig. 1) help to recognize a pattern in the observed genetic variation for *L. perenne*. No geographical pattern could be recognized in the composition of the four clusters. There were three characters that significantly separated all clusters (Table 4): length at ear emergence (NH), length at thirty days after ear emergence (HA) and number of spikelets (NS). All other characters were only important for separating the clusters on the second axis (Fig. 1) e.g. habitus (H), length of flagleaf (LF), or only on the first axis, e.g. date of ear emergence (DE), ear length (EL). Only the separation in the vertical direction (Fig. 1; axis 2) is significantly correlated with environmental factors. This indicated that although environment is important for characterizing morphologic groups within *L. perenne*, there is also a large proportion of variation not readily explained by these factors.

Correlation between environment and morphology

The general level of correlation observed between environmental factors and morphologic traits is not very high (0.41 - 0.78). In this study the explained variation varies from about 16% to 64%, indicating that the found relations should be treated with care. Another concern is the fact that these relations are correlations: they do not necessarily imply a causal relation.

The highest correlation in the present study was found between latitude and habitus (0.78): a more prostrate growth habit is mainly found at higher latitudes (the Netherlands). This does not agree with the positive correlation of Balfourier and Charmet (1991) between altitude and growth habit, as the populations from the Netherlands are from the lowest altitudes in this study. No significant correlation was observed between altitude and growth habit. This difference in result is clearly caused by the strong negative correlation (-0.72) between latitude and altitude in this study, whereas in the study of Balfourier and Charmet these factors were not so highly correlated (-0.40). Both in this study and in the study of Balfourier and Charmet (1991) a similar correlation (0.47 and 0.42) was found between latitude and date of ear emergence. This agrees with the negative correlation between altitude and heading date (-0.28) of Balfourier and Charmet.

Only significant correlations were found for temperature factors, latitude, and altitude in the present study. Precipitation did not give any significant correlation, although it is reported (Tyler et. al., 1969) as an important selective force. This may be caused by the fact that there were no populations from locations with water stress (less than 500 mm precipitation a year) in this trial. The differences in precipitation found, may have been to small to have acted effectively as a selective force.

Results from the present study show that it is difficult to find strong relations between morphological variation and environmental factors. The outcome of this kind of correlation study also depends on the

intercorrelations between the environmental factors used, as shown in the present study for latitude and altitude. Extrapolation of results to other sets of populations is therefore hazardous.

Breese and Tyler (1985) report that intensive grazing leads to prostrate, later, more persistent plants with more vegetative growth. Haying, or a combination of grazing and haying leads to more erect, earlier, less tillering plants. In this trial no management effect could be found for habitus and date of ear emergence. Grazing appeared to create plants with more spikelets and longer flag leaves. The former seems to conflict with, and the latter seems to confirm the statement of Breese and Tyler (1985) that populations from more intensively grazed grassland show more vegetative growth. The higher number of spikelets per spike formed under grazing conditions, observed in this trial, could compensate the reduced generative growth.

Effectiveness of subsampling

Subsampling of the locations did not, in most cases, result in distinct samples. Although almost all populations differed in more than one character (Table 6), in general the populations within one location did not differ. Only subsampling of the locations Neerijnen and Yde resulted in distinct samples. This can be explained by the intra-location difference in management. Neerijnen II is a grassland managed by haying three times after June 15. Neerijnen I and III are grazed grasslands. This has resulted in differences between the populations e.g. the mean date of ear emergence is two weeks earlier for Neerijnen II. The ecotype from Yde II is also different from the other two populations from this location, although this is less clear as compared to the difference found at Neerijnen. Although all grasslands at Yde are grazed and hayed, there is a difference in grazing period: May until October as opposed to half July until October. The only other location for which a difference in management was reported was Zwolle. All sub-locations at Zwolle were hayed twice a year, but one sub-location was also grazed during August and September. This did not lead to large phenotypical differences between the three populations collected. It could be that this difference in management is too small to be effective, another option is that this type of management has only been performed during a shorter number of years. Tyler and Chorlton (1976) remark it is probably crucial that the management is constant during a long period, for management type to be successful as a selective force. Generally the results indicate that for successful small scale sampling, and for the conservation of different populations within a small area, variation in grassland management is most important.

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CHAPTER 6:

ALLOZYME DIFFERENTIATION OF EUROPEAN POPULATIONS AND CULTIVARS OF *LOLIUM PERENNE* L., AND THE RELATION TO ECOGEOGRAPHICAL FACTORS

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Submitted to Euphytica

Summary

Sixty *Lolium perenne* populations were screened for allozyme diversity at five loci. Objective was to determine whether allozyme diversity could be used as selection criterion for genebank accessions of *L. perenne*. Subsampling of locations was tested with allozyme analysis to determine whether genetically different populations could be collected at one location. Correlations between allelic frequencies and environmental factors and morphological data were established, to find ecogeographical patterns in the observed variation. Results indicated that almost each allelic variant could be observed in each population screened. Very few unique alleles were found. Differences between populations were largely caused by differences in allelic frequencies. Few correlations were found with environmental factors and morphological data. For some allele frequencies a north-south cline was observed. Generally, allozymic data of the five screened loci appeared not useful for the selection of accessions for genebank storage. Significant genotypic differences between populations collected at one location could be established. In general these results agreed with earlier results concerning phenotypic variation in the same populations.

Introduction

Lolium perenne L. (perennial ryegrass) is one of the most commonly used fodder crops in the world. It is a cross-breeding species with large intra-population variation. Multiplication of populations for genebanks is space and time consuming. Some researchers have tried to find climatic, environmental and geographical patterns in the observed variation to give a scientific base to the choice of accessions for storage in genebanks. Correlations between environmental factors and agricultural traits have been reported (Balfourier & Charmet, 1991; Tyler & Chorlton, 1976) for perennial ryegrass, but in most cases relations between observed variation and environmental factors are very complex. Peeters et al. (1988) report on a case study in *Hordeum spontaneum* L. They state that simple factors, such as country of origin, are most efficient in predicting the amount of variation to be found.

An other way to improve the selection of accessions is the use of genetic markers. For instance, isozyme variation can be used to determine which populations are most variable or unique. As these markers are supposed to be selective-neutral, one can argue if they have any value for predicting variation at

agronomically important traits. There are indications that allelic forms are not selective-neutral. Rainey et al.(1987) report a correlation between Phosphoglucomutase (Pgm) and the dark respiration efficiency for *L. perenne*. Humphreys (1992) reports a relation between Acid phosphatase (Acp) and heading date and between Phosphoglucoisomerase (Pgi) and water soluble carbohydrate content for *L. perenne*. Relations between allelic frequencies and ecogeographical factors can also be of interest, to observe whether certain allelic variants are linked with environmental factors and therefore could indicate different genepools.

The objectives of the present study were: (1) to analyse the genotypic variation at several allozyme loci in a collection of European *L. perenne* populations and cultivars and to determine the distinctness of the populations, (2) to detect relations between allelic frequencies and morphological traits and ecogeographical factors (3) to determine whether sub-sampling of locations results in genotypically distinct populations.
With this information it can be established whether isozymic data are useful as rational selection criterion of perennial ryegrass populations for genetic resources purposes.

Material and Methods

Five allozymes were used to detect genetic variation in 60 *L. perenne* populations. The collection contained 21 Dutch populations, and 28 populations from various other European countries. Also 11 cultivars of perennial ryegrass were analyzed. The Dutch populations were collected (for detailed description of the sampling; Loos, 1993b), most of the European populations were obtained from the Welsh Plant breeding Station, and the German populations were obtained from the Forschungsambt der Landwirtschaft (FAL, Braunschweig). All populations are listed in Table 1. The used allozymes are listed in Table 2. Electrophoretic procedures were according to the Tris/citrate system described by Loos & Degenaars (1993). For each population 60 plants were analyzed, a population of an inbreeding *Lolium* species, *L. temulentum* L., was used as allele reference.

Data analysis

The mean number of alleles per locus (A) and the heterozygosity ($H = 1 - \Sigma p_i^2$) for each locus separately and averaged over all loci, were determined. The means and standard deviations of all alleles were calculated for each of four groups: Dutch populations, German populations, European populations and cultivars. The European group is defined as all European populations minus the Dutch and German populations.

Wright's (1965) fixation indices were calculated. F_{IT} represents the fixation of the individuals in relation to the total (all 60 populations combined) population. F_{IS} gives the fixation of individuals in relation to each sub-population (average of 60 populations). Both parameters measure the surplus/deficit of heterozygotes, and can become negative. F_{ST} is the fixation index that represents the differentiation level of the populations, and is equivalent with the G_{ST} of Nei (1977). F_{ST} can only have positive values. All three parameters are related true the formula: $(1 - F_{IT}) = (1 - F_{IS})(1 - F_{ST})$.

Table 1: Populations of *L. perenne* used for isozyme analysis

no	origin	name	number	m/e	A	H	no	origin	name	number	m/e	A	H
1	nl	'Lamora'	80.005	* -	2.4	.274	31	nl	Yerseke	90.702	* *	3.4	.288
2	da	'Hora'	80.007	* -	3.0	.238	32	nl	Yerseke	90.703	* *	3.2	.340
3	nl	'Barstella'	80.024	* -	3.0	.334	33	b	Moinet	9066.72	* *	2.6	.328
4	nl	'Perma'	80.003	* -	2.6	.366	34	f	Moutiers	9081.81	* *	1.8	.276
5	b	'Vigor'	80.023	* -	2.4	.308	35	f	Mirecourt	9113.72	* *	2.0	.356
6	nl	'Barenza'	80.016	* -	2.8	.315	36	f	Mirecourt	9110.72	* *	2.4	.233
7	gb	'Cropper'	80.059	- -	2.6	.366	37	gbw	Overton	9995.80	* *	2.8	.316
8	nl	'Elka'	80.217	- -	2.0	.333	38	gbw	Gogidan	9793.00	* *	3.0	.402
9	nl	'Premo'	80.025	- -	2.2	.364	39	gbw	Betswywith	10411.85	* *	2.2	.294
10	nl	'Barclay'	80.262	- -	2.0	.224	40	gbe	Chirkton	9957.82	* *	2.6	.404
11	nl	'Jetta'	80.348	- -	2.8	.222	41	gbe	Irby	11137.89	* *	2.8	.410
12	nl	Peize	90.101	* *	3.0	.311	42	s	Wald	10286.83	* *	2.4	.289
13	nl	Peize	90.102	* *	3.0	.324	43	s	Turbenthal	10284.86	* *	2.0	.332
14	nl	Peize	90.103	* *	2.6	.316	44	s	Ballens	9088.82	* *	2.4	.375
15	nl	Yde	90.201	* *	2.8	.323	45	s	Kippel	10288.83	- *	2.4	.280
16	nl	Yde	90.202	* *	2.6	.301	46	i	Poirino	8591.00	* *	2.6	.324
17	nl	Yde	90.203	* *	2.8	.278	47	i	Mendatica	8604.00	- *	2.0	.208
18	nl	Balkbrug	90.301	* *	3.0	.301	48	rou	Tirgu	9975.81	* *	2.2	.309
19	nl	Balkbrug	90.302	* *	3.0	.299	49	rou	Les	9984.00	- *	2.4	.328
20	nl	Balkbrug	90.303	* *	3.4	.381	50	rou	Rodnei	9981.85	- *	2.8	.298
21	nl	Zwolle	90.401	* *	2.6	.216	51	a	Hilgau	90.800	* *	2.6	.355
22	nl	Zwolle	90.402	* *	2.4	.213	52	g	Nordenham	90.901	- *	3.0	.311
23	nl	Zwolle	90.403	* *	3.2	.278	53	g	Nordenham	90.902	- *	2.8	.287
24	nl	Geldermalsen	90.501	* *	3.2	.266	54	g	Bremerhaven	90.903	- *	2.6	.289
25	nl	Geldermalsen	90.502	* *	3.0	.313	55	g	Witzenhausen	90.904	- *	2.6	.296
26	nl	Geldermalsen	90.503	* *	3.2	.307	56	g	Witzenhausen	90.905	- *	3.2	.336
27	nl	Neerrijnen	90.601	* *	2.6	.267	57	g	Lichtenau	90.906	- *	3.0	.335
28	nl	Neerrijnen	90.602	* *	2.8	.297	58	g	Schongau	90.907	- *	3.0	.366
29	nl	Neerrijnen	90.603	* *	2.6	.273	59	g	Kempten	90.908	- *	3.0	.285
30	nl	Yerseke	90.701	* *	3.2	.343	60	g	Kempten	90.909	- *	2.4	.266

nl the Netherlands, b Belgium, gbe Great Britain England, gbw Great Britain Wales, f France, s Switzerland, rou Rumania, a Austria, g Germany, i Italy da Denmark.
A= mean number of alleles per locus, H= direct-count heterozygosity, m/e morphological/environmental data available, * yes - no.

Environmental data were obtained for all populations studied except the cultivars (49 populations, see Table 1). The environmental factors used are given in Table 2, these data are generally based on thirty year averages or more (Meteorological Office, 1972; Wallen, 1970 and 1977; Rudloff, 1981). Scores for morphological characters were available for a subset of populations (42 populations, see Table 1). These morphological characters were scored in a field trial during 1991/1992 (Loos, 1993), the characters used are listed in Table 2. Spearman's rank correlation coefficient was calculated between the allelic frequencies, mean number of alleles, heterozygosity at each locus, and the environmental factors and morphological characters. Management data were available for all populations except for

Table 2: Enzymes: E.C. number, used Code and number of alleles observed, and environmental factors and morphological traits and their ranges

Enzyme and EC number	code & allele	Environmental factor & abbreviation	Range	Morphologic . factor & abbreviation	Range
Acid phosphatase	Acp	Latitude	44.55	Date of ear emergence	24.5
E.C. 3.1.3.2	4	(LAT)	53.10	(DE)	76.3
Phosphoglucoisomerase	Pgi	Longitude	3.5W	Height at ear emergence	13.9
E.C. 5.3.1.9	7	(LON)	27.0E	(NH)	28.8
Phosphoglucomutase	Pgm	Altitude	-1	Height after ear	64.4
E.C. 2.7.5.1	4	(ALT)	1840	emergence (HA)	93.4
Shikimate dehydrogenase	Sdh	Precipitation	506	Lenght of the upper	41.0
E.C. 1.1.1.25	3	(Prec)	1388	internode and ear (LNE)	61.1
6-Phosphogluconate	Pdg	Mean daily temperature	6.3	Length of the upper	26.4
dehydrogenase	4	(MDT)	12.0	internode (LN)	35.7
E.C. 1.1.1.44					
		Minimum temp. of the	-8.3	Ear length	15.9
		coldest month (minCM)	2.2	(EL)	26.8
		Maximum temp. of the	18.1	Number of spikelets	16.0
		warmest month (maxWM)	29.4	(NS)	24.8
				Number of florets	7.5
				(NF)	10.5
				Habitus	2.9
				(H)	5.7
				Length of the flag leaf	9.6
				(LF)	22.1
				Width of the flag leaf	4.3
				(WF)	6.0

the cultivars. Three categories were distinguished, only haying, haying with aftermath grazing and only grazing. With the Least Significant Difference test ($P \leq 0.01$) differences in allelic frequency between management types were tested for each allele found.

The distances between all populations, based on the allelic frequencies, were calculated using the Manhattan distance $M(x,y) = \Sigma_i |x_i - y_i|$, with x_i and y_i as the allelic frequencies of populations x and y for the i^{th} allele. The populations were scaled in a two-dimensional space using nonmetric scaling (Kruskal & Wish, 1978). This is a graphical method for representing the distances between populations. Nonmetric indicates that the rank number of the distances were used. The

Manhattan distances were also used to cluster the populations according to the unweighted pair group method average algorithm (UPGMA).

For the Dutch and German locations homogeneity of the populations within each location was tested with the Chi-square test. After pooling of the allelic frequencies of the three sub-populations from the Dutch locations, the Chi-square test was used to test for significant differences between the Dutch locations.

Results

The mean number of alleles (A) and the mean heterozygosity across all loci (H) are given in Table 1. The mean, standard deviation, minimum and maximum of the allelic frequencies for each of four groups are given in Table 3. Almost each allele is found in each group, only the Pgi-x, Pgi-e and Pgm-x alleles are found in one or two of the groups and always in a frequency of less than one percent. The means of the frequencies of each allele are comparable between the four groups, only for the Acp alleles the mean frequencies for the European group are clearly distinct. The standard deviation for the European populations group and the cultivar group is, for almost each allele, much larger than for the Dutch and German groups.

The values of Wright's fixation indices for each locus and averaged across all loci are given in Table 4. The fixation indices were lower for Pgi and Pgm compared to the other three loci. The F_{IS} for Pgm is negative indicating a heterozygote surplus at this locus. The level of between population differentiation (F_{ST}) is low.

The correlations between the allelic frequencies and the environmental and morphological data revealed several significant ($P \leq 0.01$) correlations. These correlations are given in Table 5. Pgi-c* and Acp-a show a decrease, and Acp-b an increase in frequency as the mean daily temperature rises. Acp-a and Sdh-c are more frequent in the north (LAT), whereas Sdh-b is less frequent in the north. In populations from higher altitudes more Sdh-b and less Sdh-c alleles were found. On locations with more precipitation less Acp-b alleles were found. In populations from more to the east (LON) more Sdh-b alleles were observed.

There were little significant correlations observed between morphological factors and the allelic frequencies. Later heading populations contained more Sdh-c alleles and less Sdh-b alleles. Lower number of spikelets was correlated with a higher frequency of Pgi-c alleles. Smaller height after ear emergence was correlated with more Pgm-c alleles and less Sdh-c alleles, and a smaller ear length with more Sdh-c alleles. The mean number of alleles (A) was positively related with latitude and negatively with altitude. On lower, more northern locations the number of alleles found was higher. Also the heterozygosity for Sdh was higher more to the north-west, also combined with significant correlations with maxWM, minCM and ALT. This agrees with the observation that the Sdh-c allele was mainly found in the Dutch and English populations. No significant correlations for the heterozygosity at other loci or the average heterozygosity were found.

The LSD test ($P \leq 0.01$) for differences in allelic frequencies between management types did not result in any significant difference.

Table 3: Mean frequency, standard deviation, minimum and maximum for each allele within each group

allele		Dutch	German	Europe	Cultivar	allele	Dutch	German	Europe	Cultivar
pgi-x	M	0.08	-	0.54	-	acp-a	70.21	69.41	44.25	63.35
	SD	0.24	-	2.36	-		8.35	15.40	17.61	24.55
	Min	0.00	-	0.00	-		53.30	46.40	20.90	0.00
	Max	0.80	-	10.30	-		85.80	84.20	77.20	85.60
pgi-a	M	38.46	39.81	35.48	36.51	acp-b	23.43	23.22	37.24	29.75
	SD	5.55	6.40	16.35	17.13		6.43	11.30	19.83	26.28
	Min	28.30	33.00	0.00	6.30		11.30	12.70	0.00	1.70
	Max	50.00	51.80	70.60	65.00		33.10	41.10	78.70	98.20
pgi-b	M	49.69	47.58	52.21	51.67	acp-c	3.49	4.49	9.64	2.81
	SD	5.75	5.14	18.40	16.90		2.66	3.38	16.77	6.35
	Min	36.40	40.40	19.80	18.90		0.00	0.00	0.00	0.00
	Max	57.60	55.70	95.60	78.90		10.20	9.40	73.50	21.70
pgi-c	M	5.71	5.72	6.08	4.03	acp-d	2.85	2.88	8.46	4.09
	SD	5.08	3.25	8.94	7.35		2.18	3.30	9.95	6.06
	Min	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00
	Max	19.20	9.60	30.20	23.50		5.90	10.00	30.60	18.30
pgi-c*	M	5.85	5.77	5.23	7.48	sdh-a	5.38	1.59	3.99	6.29
	SD	5.08	3.25	8.94	7.35		7.25	2.03	6.67	7.73
	Min	1.40	0.90	0.00	0.00		0.00	0.00	0.00	0.00
	Max	12.30	13.40	26.20	17.50		30.60	6.40	21.90	24.40
pgi-d	M	0.17	1.13	0.47	0.31	sdh-b	89.09	97.18	93.77	86.96
	SD	0.48	1.05	1.21	0.69		7.87	2.05	7.58	11.53
	Min	0.00	0.00	0.00	0.00		64.80	92.70	78.10	59.00
	Max	2.00	3.10	4.30	1.70		98.20	100.00	100.00	100.00
pgi-e	M	0.04	-	-	-	sdh-c	5.54	1.23	2.24	6.75
	SD	0.20	-	-	-		5.05	1.05	5.20	6.67
	Min	0.00	-	-	-		0.00	0.00	0.00	0.00
	Max	0.90	-	-	-		16.90	3.10	17.50	20.30
pgm-x	M	0.04	-	-	-	pdg-x	0.09	-	-	-
	SD	0.17	-	-	-		0.39	-	-	-
	Min	0.00	-	-	-		0.00	-	-	-
	Max	0.80	-	-	-		1.80	-	-	-
pgm-a	M	69.16	61.33	58.04	67.52	pdg-a	2.47	0.28	0.85	0.15
	SD	6.44	3.85	15.68	18.85		5.09	0.83	2.01	0.51
	Min	59.20	55.40	36.70	34.10		0.00	0.00	0.00	0.00
	Max	84.50	66.10	90.80	92.50		23.70	2.50	8.50	1.70
pgm-b	M	28.72	37.09	39.78	29.95	pdg-b	97.10	99.42	98.80	99.70
	SD	5.65	4.03	15.43	17.90		5.09	1.15	2.11	0.67
	Min	14.70	32.0	9.20	7.50		76.30	97.30	91.50	98.30
	Max	36.70	60.0	63.30	65.10		100.00	100.00	100.00	100.00
pgm-c	M	2.09	1.58	2.18	0.53	pdg-c	0.34	0.30	0.34	0.15
	SD	2.00	1.67	3.06	1.01		0.67	0.90	0.83	0.48
	Min	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00
	Max	7.60	4.00	11.30	2.60		2.00	2.70	3.30	1.60

Table 4: Wright fixation indices for 60 *L. perenne* populations

Locus	F_{IT}	F_{IS}	F_{ST}
Acp	.234	.096	.153
Pgi	.083	.019	.065
Pgm	.047	-.028	.073
Sdh	.255	.182	.090
Pdg	.744	.740	.014
Mean	.136	.045	.096

The result from the nonmetric scaling of the distances between the populations is presented in Figure 1. This figure shows that the European populations and the cultivars are scattered all over the plot. The Dutch and German populations are localised in a much smaller area of the plot, indicating that Dutch and German populations have relatively small distances to each other and relatively large distances to the other populations from this trial. The European populations and the cultivars have relatively large distances between each other. Clustering of the populations, based on the same distances, did not result in clearly interpretable clusters. Most of the European populations and the cultivars formed single population clusters, leaving all Dutch, German and some European populations and some cultivars in one very large cluster. Changing of the cluster algorithm (single linkage, average linkage within groups, furthest neighbour) did not enhance the results.

Table 5: Spearman rank correlation coefficients

Allele	Factor type							
Pgi-c*	environmental	MDT	- 0.49					
Pgi-c	morphologic	NS	- 0.56					
Acp-a	environmental	LAT	0.48	MDT	0.52			
Acp-b	environmental	MDT	0.55	Prec	- 0.38			
Pgm-c	morphologic	HA	- 0.42					
Sdh-b	environmental	maxWM	0.51	LAT	- 0.46	LON	0.47	ALT 0.49
	morphologic	DD	- 0.55					
Sdh-c	environmental	maxWM	- 0.59	LAT	0.53	ALT	- 0.50	
	morphologic	DD	0.59	HA	0.40	AL	0.43	

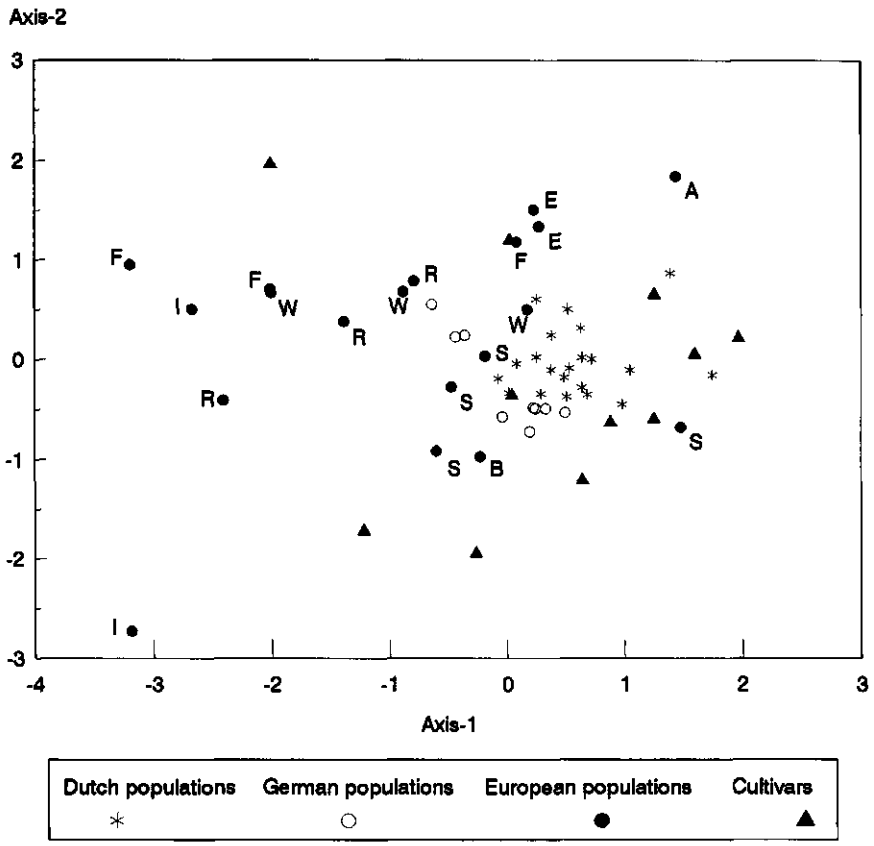

Figure 1: Scatterplot of 60 *L. perenne* populations, based on the Manhattan distances calculated from the allelic frequencies for five allozymes. For the European populations the following abbreviations were used: a Austria b Belgium e England f France i Italy r Rumania s Switzerland w Wales.

The Chi-square test for homogeneity within the Dutch locations resulted in several non homogenous locations, results are given in Table 6. The populations of location Peize and Yerseke showed no significant difference at any locus studied. The populations at the other locations showed significant differences at, at least, one locus. Pgm was the allozyme that showed the most significant differences between populations within locations. Pgi and 6-Pdg hardly showed any significant differences between populations from one location.

The Chi-square test of the allelic frequencies between the Dutch locations showed several significant differences, which are tabulated in Table 7. For four of the five enzymes studied (Pgi, Acp, Pgm and Sdh), three to four groups could be distinguished, although these groups were in most cases overlapping. For 6-Pdg only one group could be distinguished, as this locus was nearly monomorphic. The population from Yerseke

Table 6: Chi-square test for differences in allelic distribution within locations

locus	P	Yd	B	Z	G	N	Ye
Acp	10.17	18.31	12.22	8.23	8.41	26.04	6.53
	n.s	**	n.s	n.s	n.s	**	n.s
Pgi	6.00	12.52	12.10	9.64	2.93	22.22	18.92
	n.s	n.s	n.s	n.s	n.s	**	n.s
Pgm	10.19	13.52	18.04	8.44	19.41	8.04	2.93
	n.s	**	**	n.s	**	n.s	n.s
Sdh	10.81	8.65	2.42	43.89	1.27	14.28	6.54
	n.s	n.s	n.s	**	n.s	**	n.s
6-Pdg	-	2.01	-	-	4.47	-	4.60
	-	n.s	-	-	n.s	-	n.s

** $P \leq 0.01$, n.s not significant, P Peize, Yd Yde, B Balkbrug, Z Zwolle, G Geldermalsen, N Neerijnen, Ye Yerseke

differs most from all other Dutch locations, as the population from Yerseke is significantly different at the Pgi and Sdh locus from all other Dutch populations.

Tabel 7: Chi-square test for differences between the Dutch locations

	Location						
Locus	P	Yd	B	Z	G	N	Ye
Pgi	ab	b	ab	ab	ab	a	c
Acp	a	ab	b	a	bc	d	dc
Pgm	abc	b	abc	a	c	ab	abc
Sdh	c	c	c	d	b	b	a
6-Pdg	a	a	a	a	a	a	a

Similarity in alpha indicates no significant differnce at $P \leq 0.01$

Discussion

In general all populations contain the same alleles for all five allozymes. Only few unique alleles were detected, most alleles can be characterized as common widespread alleles (frequency $>10\%$, more than 2 regions; Brown, 1978). Only few alleles (Pgi-e, Pgm-x, Pdg-x) are rare and localized (frequency $<10\%$, one region; Brown, 1978), and one allele (Pdg-c) is rare, widespread (frequency $<10\%$, more than 1 region; Brown, 1978). The variation in number of alleles and the heterozygosity is small and could only be linked with few environmental data. Differences are too small to serve as reliable selection criterion for accessions. Hamrick & Godt (1990) report for outcrossing wind-pollinating

species an A of 1.21 and a H of 0.162, based on 105 taxa with a mean of 10.7 populations and 16.7 loci analyzed per taxon. The mean of both parameters were substantially higher in this study (A=2.7, H=0.308), indicating that *L. perenne* is a species with extremely large intra-population variation. Part of these large values for the parameters A and H, is due to the fact that none of the loci studied was monomorphic. Charmet et al.(1993) report diversity statistics of A=2.75 and H=0.270, based on 60 French perennial ryegrass populations and 7 polymorphic isozyme loci. These diversity statistics are similar to the ones in the present study. In an earlier study populations from the other two cross-breeding *Lolium* species were determined , using the same enzymes (Loos, 1993a). For *L. rigidum* Gaud. A= 3.3 and H= 0.336, for *L. multiflorum* Lam. A= 3.2 and H= 0.363. These diversity statistics are somewhat higher as for *L. perenne* populations, indicating that both species are closer to the Mediterranean centre of origin of the genus *Lolium*, than perennial ryegrass.

The positive mean for the within population fixation index (F_{IS}, Table 4) indicates that in general there is a deficit of heterozygotes within the populations. The mean value F_{IS} of 0.045 is comparable with values reported by Hayward & McAdam (1977) for *L. perenne* cultivars and Charmet et al. (1993) for French perennial ryegrass populations. The value of 0.096 for the mean F_{ST} is comparable with the value of 0.099 reported by Hamrick & Godt (1990) for outcrossing, wind-pollinated species. Between the loci studied differences appear in the F_{ST} values, Acp has the highest value which means that between population differentiation is most developed at this locus.

Correlations between allelic frequencies and morphologic and environmental data were not very strong. No relation between Acp and date of ear emergence was found as earlier reported by Humphreys (1992). The only alleles for which a reliable north-south cline could be found were Acp-a, Sdh-b and Sdh-c, as these alleles were not only correlated with latitude but also with other factors which are related to the latitude as mean maximum temperature of the warmest month, mean daily temperature and altitude (Table 4). This agrees with Charmet et al. (1993) who report a north-south cline for Acp-a and Acp-b in a collection of French perennial ryegrass populations. Arcioni et al. (1988) found a correlation between the Pgi-b and Pgi-d alleles and the latitude. This study was based on only Italian *L. perenne* populations. This correlation could not be confirmed neither with the present results nor with the results from Charmet et al. (1993).

The mean and standard deviation of each allele for all four groups (Table 3) clearly shows that the allelic variants observed are nearly identical for all groups. The larger standard deviation for the European populations for several alleles, is related to the larger area covered by the European populations. The larger variation of the allelic frequencies within the European populations is also demonstrated in Figure 1, by the scattered position of these populations.

The localization in the plot (Figure 1), the large standard deviations and spread minimum and maximum values for the allelic frequencies (Table 3), the mean number of

alleles and the heterozygosity (Table 1) of the cultivars show absolutely no reduction in variation compared to the collected populations. Morphologic analysis of six of these cultivars (Loos, 1993b: Table 1) showed that the phenotype of all these cultivars consisted of later heading, more erect growing plants which were taller at ear emergence. They therefore were a distinct group phenotypically. The absolute lack of allozyme variation reduction, indicates that the screened allozymes are indeed selective-neutral.

For the Dutch populations Figure 1 illustrates that distances between the Dutch populations are relatively small. This indicates that the same alleles were found in all Dutch populations in similar frequencies, possibly due to a specific balancing selection. The same holds for the German populations, although they seem to split into two groups. Charmet et al. (1993) call the existence of this kind of balancing selection unlikely, as it should be of a very sophisticated nature due to the polymorphism at most loci and the selective-neutral theory for allozymes.

The Chi-square test showed (Table 6) that at location Neerijnen most differences were observed between the three populations. This agrees with earlier results (Loos, 1993b) that Neerijnen was the most variable location sampled within the Netherlands for morphologic characters. Also the fact that location Peize and Yerseke do not show any significant difference agrees with earlier morphologic comparison (Loos, 1993b), these locations were very homogenous.

The Chi-square test between Dutch locations shows that Yerseke (Table 7) is the most particular location within the Netherlands. In comparison high levels of Pgi-c and Sdh-c alleles and low levels of the Acp-a allele were found at this location. This does not agree with the morphological variation found here. Morphologically populations from Yerseke were hardly distinguishable from populations from more northern inland locations. It was concluded that the permanent salt stress at Yerseke, was not a selective force on the morphology. It appears that this salt stress does influence the frequency of allelic variants (population structure) of populations, possibly indicating a physiological adaption to the saline environment. This does not agree with the selective-neutral theory for allozymes.

Generally, all allelic forms can be detected in a very small number of populations, only allelic frequencies show large differences between populations. The danger of losing allelic forms due to the small number of collections is not present. Results also show that it is not possible to choose between populations based on allelic frequency data for genetic resources purposes. The number of alleles and the heterozygosity of all tested populations are too similar to make a reliable decision. This is illustrated by the cultivars which do not show any reduction for allelic variation, although morphologically they constitute a specific group. Large differences in allelic frequencies can be observed, but these frequencies are not or too weakly linked with environmental factors and morphologic data, to make a meaningful selection of accessions. Allozymic data can be used as an indicator of genetic differences between sub-samples from one location. The results for the Dutch populations indicate that *in situ*

conservation of perennial ryegrass can be an option, but that the sites should be chosen throughout Europe to cover all differences in population structure.

The results for the cultivars show that also *ex situ* storage of accessions can cover the whole spectrum of alleles and allelic frequencies.

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GENERAL DISCUSSION

Several aspects of variation within the genus *Lolium*, and more in detail within *Lolium perenne* (perennial ryegrass) have been highlighted. As the results are extensively discussed in each chapter, the general discussion is focused on two aspects of the research.

Speciation

It is clear that the genus *Lolium* is a very variable genus. The variation within the species reduces the clarity of separation of the species. Stebbins (1956) found the differences between *Lolium* and *Festuca* not sufficient to justify two separate genera. He also states that the family of *Poaceae* is a phylogenetically derived family, and therefore of comparatively recent origin. Differences between species and between genera are still developing. In older families, intermediate forms or species have become extinct, therefore genera and species delimitations are clearer in these families, e.g. the *Papaveraceae* (Stebbins, 1956). Producing additional information besides morphological data, such as cytogenetic studies and biochemical studies, is of little use for these families as genera and species delimitations are easily made with simple morphological characters. In families of more recent origin, such as the *Poaceae*, as many characteristics as possible should be used to establish the relationships between the species. In Chapters 2, 3 and 4 several types of characters have been analyzed for the genus *Lolium*. No phylogenetic analysis was performed with these data, only phenetic analysis due to the nature of the characters used. Results indicate that all species, as mentioned in the general introduction, can be recognized, although species delimitations are not unambiguous. Only for *L. persicum* and *L. temulentum* the results indicate that these species could possibly be two varieties of one species. All species show diagnostic characters for one ore more of the different type of characters. Although the overlap of species is often unsatisfactory, joining of species would be even more artificial. As speciation is a continuous process it cannot be predicted at which point in time species are going to be sufficiently delimited.

Man has had large influence on the speciation within *Lolium*. This is illustrated by the three weedy species within the genus. *L. remotum* is known as a weed in flax (Hjelmqvist, 1950), *L. temulentum* and *L. persicum* are known as weeds in cereals (e.g. Dore, 1950). All three are mimicry weeds, the morphology of the seeds and/or the habit of the plant is similar to the crop in which it grows. Until a few decades back, these three species had a significant impact as weeds, but due to enhanced seed cleaning techniques the distribution area of these species has largely decreased (Hubbard, 1954).

Other examples of the influence of man on the genus *Lolium*, are the species *L. perenne* and *L. multiflorum*. Their distribution area has largely increased due to sowing by man. Scholz (1975) stated that man has had an

enormous influence on the development of both species. According to Scholz (1975), this influence started no more than a few thousand years back, with the cutting and burning of forest for replacement by grassland for cattle, and the discovery of hay making. This has made it almost impossible to determine in which parts of the world both species are indigenous. Not only the distribution area of both species is influenced by man but also the phenotype. Selection changes the phenotype in favour of character states desired by man, such as increased yield. Tyler (1979) observed that after a period in which the standard of management is relaxed, natural phenotypes reoccur. This is confirmed with the results from Chapter 5: Dutch perennial ryegrass populations, collected after a period of more relaxed management, have a distinct phenotype compared to cultivars. Tyler (1979) also indicated that the differences between wild and cultivated forms are extremely blurred for *L. perenne*. This statement is confirmed by the results from Chapter 6: for allozymic variation, cultivars show absolutely no reduction in variation compared to natural populations. Ellenberg (1963) calls the type of plant as *L. perenne* semi-domesticated, as the crop is not harvested each year but only kept at an acceptable production level using reseeding. During each phase of their life-cycle, populations are exposed to selection forces. Leading to the situation that in grasslands cultivars are often mixed with plants that have been exposed to environmental selection for a number of years. This makes the distinction between natural and cultivated grassland extremely vague.

Chapter 5 illustrates, as management is the factor that optimizes the amount of genetic variation found within a location, the enormous influence man has had and still has on the amount of variation in phenotypes of *L. perenne*. Reduction of the influence of man would probably lead to the existence few differing perennial ryegrass phenotypes, and could in some areas even mean extinction of perennial ryegrass. In the Netherlands, foreland and salt marshes are the only original habitats for grassland (Bink et al., 1984). Although *L. perenne* is a species with much competitive ability, it would suffer from a large reduction of distribution area in case management of grasslands was totally abandoned. Because mainly under man-made conditions, e.g. fertilizing, treading, intensive grazing, drainage, *L. perenne* expresses this competitive nature.

For *L. rigidum* the influence of man is less strong. *L. rigidum* is used in some parts of the world (e.g. Australia) as a cultivated fodder crop, but in Europe this is not current. In Europe the fate of *L. rigidum* depends on the perspectives of *L. rigidum* as a fodder crop in dry areas or as a crossing parent in breeding programmes. Next to its presence in cultivation *L. rigidum* is well capable to maintain itself under less cultivated circumstances, this in contrast to, especially, *L. perenne*. Hartley (1956) states that *L. rigidum* originates from the Mediterranean region and that *L. perenne* and *L. multiflorum* originate from the Eurasian region. The ancestral species of the genus *Lolium* is supposed to have originated in the Mediterranean region (Malik, 1967). This would indicate that *L. rigidum* could be the wild form for both cultivated species. The relation between wild and cultivated is often confirmed by the reduction of genetic variation

within the cultivated forms. Brown (1978) mentions two examples, based on allozyme variation, for which this assumption is valid. *Lycopersicon pimpinellifolium* has 61% unique allelic forms compared to those in *L. esculentum*. Both species share 37% of the allelic variants and 2% is unique for *L. esculentum*. *Oryza perennis* has 47% unique peroxidase alleles, and 22% unique esterase alleles, compared to *O. sativa*. Both species share 53% and 78% of the alleles respectively. The results from Chapter 3 do not indicate a reduction in allelic variation within *L. perenne* and *L. multiflorum*, compared to *L. rigidum*. This indicates that, if *L. perenne* and *L. multiflorum* indeed did arise from *L. rigidum*, this speciation is of recent origin. Phylogenetic relations between species cannot be determined on basis of these data.

L. loliaceum is not known as a weed nor as a crop plant, it mainly grows under poor and maritime conditions. The influence of man on populations of this species is not large, therefore it is not likely that this species becomes extinct nor that its distribution area will suddenly increase. Phenotypic developments are expected to be gradual and slow.

For *L. canariense* the same holds true as for *L. loliaceum*, it is not a crop nor a weed and grows under poor conditions, making it a stable and localized species.

The screening of the *Lolium* species for allozymic variation, added little to the species determination within the genus *Lolium*. The pattern of allozyme diversity could hardly be linked with taxonomic classification (Chapter 3); mainly because all allelic variants were common in each population screened. As pointed out in the discussion of Chapter 3, this maybe caused by the small number of enzyme systems screened. A question that can be asked is whether increase of the number of allozymes could lead to better results for genotypic screening. In Chapters 3 and 6 the calculated diversity statistics for the cross-breeding *Lolium* species were above the average for other wind-pollinated cross-breeding species (Hamrick & Godt, 1990). These statistics indicated that a larger proportion than average of the loci screened were polymorphic, and also that the average heterozygosity of the loci was far above the mean. Extension of the number of loci screened would therefore most likely result in finding monomorphic loci or less variable polymorphic loci and would not enhance the results. In literature, analyses of *L. perenne* populations for several other allozymes are reported. These allozymes are Glutamate-oxaloacetate-transaminase (GOT, Hayward & McAdam, 1977; Arcioni et al., 1988; Charmet et al., 1993), Isocitrate dehydrogenase (IDH: Lallemand et al., 1991; Charmet et al., 1993), Peroxidase (PRX: Charmet et al., 1993) and Superoxide dismutase (SOD: Charmet et al., 1993). All authors report results that confirm the expectation that higher number of allozymes screened do not improve the elucidation of speciation. Again, the within-population variation is too large compared to the between-population variation.

Another option would be to make use of molecular markers, e.g. restriction fragment length polymorphism (RFLP). Few reports are known for *Lolium* species, using these techniques. Darbyshire & Warwick (1992) report on the results for one *L. perenne*

population, which was compared with several other grass populations classified in 26 *Festuca* species and the genera *Vulpia*, *Poa* and *Puccinella*. Eleven restriction endonucleases and twelve restriction fragments from chloroplast DNA of *Petunia hybrida* Vilm. were used in this analysis. In total 341 bands were observed of which 108 (31.7%) were polymorphic. Of these 108 bands, 34 were detected in the *L. perenne* population. Only one plant was analyzed from each population. Chloroplast DNA variation in other *Lolium* species (Lehväslaiho et al., 1987; Soreng et al., 1990) is only reported for one *L. multiflorum* population, using five restriction-enzymes and direct end labelling. Again only one plant has been analyzed and compared with a large set of populations and genera mainly from the family *Poaceae*. *L. multiflorum* differs in 11 bands on a total of 144 shared bands with *Festuca pratensis*. Only one report is known (Wu et al., 1992) on the between-population variation within *L. perenne* for RFLP's. Five cultivars of perennial ryegrass were screened, using 2 restriction enzymes and 37 probes from *Festuca pratensis*. Twenty-four (65%) of these probes hybridized, resulting in on average 69% polymorphism between the five cultivars. On average 3.2 different banding patterns were observed for each restriction enzyme-probe combination. Again only one plant was analyzed for each population.

No reports on the between-species and the within-population variation are known for any of the *Lolium* species.

The results from Chapter 3 and Chapter 6 indicate that substantial variation is found within populations of the cross-breeding *Lolium* species, which makes results based on only one plant per population unreliable (Wu et al, 1992). It remains necessary to analyse a minimum number of plants for the cross-breeding *Lolium* species, unless an acceptable bulk sample can be taken. This would be desirable as otherwise the cost and time needed to analyse a population using molecular markers could be limiting. The danger of using a bulk sample would be that no differences between populations and even between species can be observed (as would be the case for a bulk sample when screening for allozyme variation). Screening of five enzyme systems resulted in a maximum of 10 bands observed (13, if the heterozygous bands were also counted), in case a plant was heterozygous (maximum variation) for each enzyme. This is a much better result as reported by Darbyshire & Warwick (1992), 34 bands out of 132 restriction enzyme-probe combinations. It is equal to the theoretical maximum number of bands reported by Wu et al. (1992), 96 bands in case of heterozygosity at all 48 restriction enzyme-probe combinations. The preliminary conclusion would therefore be that RFLP analysis will not greatly enhance the distinction of cross-breeding *Lolium* species and populations.

For the inbreeding *Lolium* species the analysis of few plants is sufficient. The observed variation would probably increase compared to the observed allozyme diversity (Chapter 3: fixation for four of the five enzymes), as the number of possible markers would greatly increase using RFLP's. The use of molecular markers for the screening of inbreeding *Lolium* populations would therefore be a valuable extension of the knowledge on these species.

Genetic resources: *in situ* conservation

In the general introduction three research questions were mentioned, concerning the genetic resources of *L. perenne*. What are the answers to these questions after analyzing the results from four years of research? Firstly, the Dutch populations do form, morphologically, a distinguishable group of forms within the genetic variation for perennial ryegrass. This conclusion is based on the observation of morphological characters only, as the heritability of these characters is better determined than for agronomically important traits as winter hardiness, spring growth etc. The date of ear emergence is one of the most important characters, both agronomically and for the recognition of breeders rights. Results indicate that genetic variation for this character is substantial in the Netherlands: in natural surroundings, populations varying from very early till very late heading could be collected. It is expected that if the Dutch populations show this amount of genetic variation for morphological characters, results can be analogous for agronomical characters. The morphological variation found within the Dutch populations is not comparable with the variation found in the cultivars used in these trials. Although date of ear emergence indicates that some Dutch populations are heading as late as the cultivars, morphologically they are distinct. The Dutch populations are a.o. more prostrate growing and shorter at ear emergence, this could indicate that this phenotype is natural for *L. perenne* in the Netherlands. The fact that Dutch populations are morphologically clearly distinct from the cultivars and that there was also substantial variation observed between Dutch populations, indicates that *in situ* conservation is a realistic option for *L. perenne*. Weibull (1989) gives several advantages and disadvantages for the *in situ* conservation of forages. The advantages are: continued co-evolution of populations and the possibility to study the ecology of the species. It is also possible to make successive collections, and it avoids space and time consuming activities for storage en regeneration for genebanks. A combination of the genetic resources conservation objective with other objectives like nature conservation could be an option. This possibility is clearly illustrated with the present results, as all Dutch populations were collected in areas managed by nature conservation organisations.
Disadvantages are that it is difficult to determine how many sites, and which sites should be preserved to optimize genetic variation. Natural populations are vulnerable to external factors, such as human influences and extreme weather conditions. Also the costs of the maintenance of conservation sites maybe high, and access of breeders can be a problem in case of a combination with nature conservation objectives.

For the allozyme variation no differences between the Dutch populations and the cultivars were found. Allelic variants were very common in all populations, the cultivars showed much larger differences in allelic frequencies than the Dutch populations. *In situ* conservation would be very successful in retaining genetic diversity at the allozyme level. The data were not useful for selection of accessions for genebanks. Phillips et al. (1993) reported for *Avena sterilis* L. (inbreeder), the wild progenitor of *A. sativa* L., the possibility to separate populations in six different groups

based on 23 loci. Selection of genebank accessions can be facilitated using these six groups, combined with morphological data.

Francisco-Ortega et al. (1992) observed for *Chamaecytisus proliferus* (L. fil.) Link a totally different pattern. Morphologically this species can be separated into seven subspecies, which are morphologically distinct and ecologically each occupy a distinct niche. Allozyme diversity shows no differentiation between these seven subspecies.

Just like in the genus *Lolium*, almost all allelic variants are common and widespread, and the within-population variation is very large. Also in this case allozyme data were considered of no use for the selection of genebank accessions.

Generally, the usefulness of screening for allozyme variation varies substantially. Compatibility behaviour and age of the genus/species are the major factors, explaining the value of this kind of data.

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SAMENVATTING

De genetische variatie binnen het genus *Lolium* werd bestudeerd, naar aanleiding van twee onderzoeksvragen. De eerste onderzoeksvraag betrof de taxonomie en de classificatie binnen het genus *Lolium*. Uit de literatuur blijkt dat de soortsbegrenzing binnen dit genus vaak onduidelijk is. Dit heeft twee oorzaken: ten eerste zijn de kruis-bevruchtende soorten onderling goed kruisbaar, hetgeen tot introgressie kan leiden. Daarnaast zijn populaties binnen dit genus sterk adaptief. De morfologische variatie en biochemische variatie binnen en tussen de diverse soorten van het genus werd geanalyseerd. De consequenties voor de classificatie binnen het genus werd bepaald. Tevens werden de huidige onderzoeks-resultaten vergeleken met gegevens uit de literatuur.

De tweede onderzoeksvraag betrof de genetische diversiteit van *Lolium perenne* L. (Engels raaigras) binnen Nederland. Engels raaigras is met circa 1,3 miljoen ha produktie grasland het cultuurgewas met het grootste areaal in Nederland. Jaarlijks wordt circa 10% van het grasland heringezaaid of doorgezaaid, daarnaast wordt Engels raaigras zeer algemeen gebruikt in o.a. wegbermen, gazons en sportvelden. Door het intensieve gebruik van Engels raaigras cultivars rees de vraag of het verzamelen van populaties Engels raaigras voor het Centrum van Genetische Bronnen in Nederland zinvol is, en of *in situ* conservatie een mogelijke optie is voor het conserveren van de genetische diversiteit van Engels raaigras. Hiervoor werd een analyse van de genetische diversiteit voor *L. perenne* L. (Engels raaigras) in Nederland gemaakt, en vergeleken met de variatie in populaties uit andere Europese landen en een set cultivars, dit zowel op morfologisch als op biochemisch niveau.

In de algemene inleiding wordt de meest recente revisie van het genus *Lolium*, door E.E. Terrell in 1968 gepubliceerd, nader besproken. Dit proefschrift gaat uit van de soortsindeling zoals deze door Terrell werd beschreven, met een aantal aanpassingen. De soorten *L. perenne* L., *L. multiflorum* Lam., *L. rigidum* Gaud., *L. remotum* Schrank, *L. temulentum* L., *L. persicum* Boiss. & Hohen. en *L. canariense* Steud. worden erkend. Daarnaast wordt de variëteit *L. rigidum* var. *rottbollioides* Heldr. ex. Boiss. samengevoegd met de soort *L. subulatum* Vis. en ondergebracht in de soort genaamd *L. loliaceum* Hand-Mazz. Analyse van Terrell's beschrijving en herbariummateriaal wijzen er namelijk op dat het onderscheid tussen beide taxa louter gebaseerd is op een verschil in grootte. Dit verschil in grootte zou o.a. veroorzaakt kunnen worden door het verschil in habitat. *L. rigidum* var. *rottbollioides* wordt, volgens Terrell, voornamelijk in zeer voedselarme maritieme habitats gevonden. De variëteit *L. rigidum* var. *rigidum* komt te vervallen.

In hoofdstuk 1 wordt de typificatie van de twee Linneaanse *Lolium* soorten beschreven: *L. perenne* en *L. temulentum*. Dit vond plaats in het kader van het Linnean Plant

Name Typification project. Het bleek dat de typificatie voor *L. perenne*, LINN 99.1, zoals voorgesteld door E.E. Terrell in 1968, goed is en gevolgd moet worden. Uit het onderzoek naar de mogelijke type exemplaren werd duidelijk dat hoewel Linnaeus de ongenaaldheid van *L. perenne* als diagnostisch kenmerk gebruikte, er in door hemzelf gedetermineerd herbarium materiaal ook genaalde *L. perenne* wordt gevonden. Voor *L. temulentum* werd het type exemplaar opnieuw gekozen. Het exemplaar aangewezen door Terrell was niet als een *L. temulentum* exemplaar gedetermineerd door Linnaeus. Bovendien was het niet aantoonbaar in het bezit van Linnaeus ten tijde van het schrijven van *Species plantarum*. Het nieuwe type exemplaar bevindt zich in het Burser herbarium in Uppsala (UPS-BURS): Vol. I, fol 113.

In hoofdstuk 2 worden 51 *Lolium* populaties, verdeeld over 7 soorten, geanalyseerd op hun variatie in morfologische en fenologische kenmerken. Dit resulteerde in twee groepen: één groep bevatte de soorten *L. temulentum* en *L. persicum*. Beide soorten waren moeilijk te onderscheiden en vertoonden sterke intergradatie. Daarnaast bestond er een andere groep, met daarin de drie kruisbevruchtende soorten: *L. perenne*, *L. multiflorum* en *L. rigidum*. Alle drie soorten zijn nauw verwant maar onderscheidbaar. De twee overgebleven soorten: *L. remotum* en *L. loliaceum* vormen geen groep. *L. loliaceum* neemt een aparte positie in binnen het genus, en lijkt het meest verwant met *L. rigidum*. *L. remotum* is min of meer intermediair tussen de kruisbevruchters groep en de *L. persicum/L. temulentum* groep. Vergelijking van deze resultaten met gegevens bekend uit de literatuur, leidde tot de vaststelling dat het gebruik van verschillende typen kenmerken resulteert in evenzoveel verschillende conclusies, hoewel niet duidelijk verschillend in de grote lijnen. Het meest waarschijnlijk is dat het genus van recente oorsprong is, waardoor de differentiatie
tussen soorten nog niet optimaal is.

In hoofdstuk 3 wordt de variatie in allozymen beschreven voor een deelset van de populaties uit hoofdstuk 2. Het bleek dat voor de kruisbevruchtende soorten, de allozymdata konden dienen als kenmerk voor de soortsindeling. Voornamelijk door grote verschillen in frequenties van dezelfde allelen en in mindere mate door de aanwezigheid van specifieke allelen. De binnen-populatie variatie was, in verhouding tot andere kruisbevruchtende soorten, zeer groot. De zelfbevruchtende soorten konden niet geclassificeerd worden met behulp van allozymdata. Voor 4 van de 5 gebruikte allozymen waren alle zelfbevruchtende populaties gefixeerd voor hetzelfde allel. Alleen voor Pgi bestond er variatie in allelen tussen de populaties, ook dit allozym was binnen iedere populatie gefixeerd.

In hoofdstuk 4 worden alle populaties uit hoofdstuk 2 samen met enkele *L. hybridum* Hausskn. (de hybride tussen *L. perenne* en *L. multiflorum*) populaties en één *L. canariense* populatie geanalyseerd, voor morfologische en biochemische zaadkenmerken. Het bleek dat wanneer de gemeten waarden van de morfologische zaadkenmerken per soort gemiddeld werden, alle soorten goed te onderscheiden waren. Het bleek ook dat, met

name binnen de kruisbevruchtende soorten, individuele populaties dusdanig ver van dit gemiddelde konden afliggen dat identificatie van populaties een moeilijke zaak blijft. Alle 7 gemeten kenmerken bleken bovendien dusdanig gecorreleerd, dat meting van twee kenmerken vaak al het optimale onderscheid gaf. Voor het zaadeiwit patroon bleek dat op grond van deze patronen de meeste populaties goed naar soort te classificeren waren. Op 52 geanalyseerde populaties waren er slechts 3 paren die een identiek bandenpatroon vertoonden. De *L. hybridum* populaties verdeelden zich, zowel op de morfologische zaadkenmerken als voor het zaadeiwit patroon, over de twee kruisingsouders *L. perenne* en *L. multiflorum* en konden niet als aparte soort geclassificeerd worden. *L. canariense* bleek zowel qua morfologische zaadkenmerken als voor zaadeiwit patroon duidelijk van de andere *Lolium* soorten te onderscheiden. Weer bleek dat *L. persicum* en *L. temulentum* moeilijk van elkaar te onderscheiden waren, ditmaal op zaadeiwit patroon. De morfologische zaadkenmerken lieten wel een duidelijke tweedeling zien, die niet altijd met de soortsindeling overeen stemde. Tevens bleek dat populaties die eerder op plantkenmerken (hoofdstuk 2) grote overeenkomst vertoonden, dit niet voor de zaadkenmerken hoefden te doen (en vice versa). Daar beide soorten mimicry onkruiden zijn in granen, zou verondersteld kunnen worden dat ze tot één en dezelfde soort behoren. Afhankelijk van het soort graan waartussen de populatie zich ontwikkelt zou er vervolgens adaptie van, met name, de morfologische zaadkenmerken optreden.

Het tweede gedeelte van het onderzoek betreft de genetische diversiteit voor Engels raaigras in Nederland. In hoofdstuk 5 wordt de analyse van 21 Nederlandse populaties, 14 Europese populaties en 6 cultivars, voor morfologische kenmerken beschreven. Daarnaast wordt de geobserveerde variatie gekoppeld aan diverse klimatologische en standplaats gegevens. Resultaten geven aan dat de Nederlandse populaties een karakteristiek fenotype hebben in vergelijking met de andere populaties uit de proef. Ze hebben o.a. een meer liggende groeiwijze, zijn kleiner bij doorschieten en hebben kortere en minder brede vlagbladeren. Opvallend is dat voor andere kenmerken o.a. doorschietdatum en hoogte na doorschieten, de variatie binnen de Nederlandse populaties net zo groot is als in alle andere populatie tezamen. Koppeling van de morfologische variatie aan omgevings en standplaats factoren leverde weinig hoge (> 0.75) correlaties op. Correlaties tussen de gebruikte omgevings- en klimaatsfactoren onderling, bemoeilijkten de interpretatie van de resultaten. Bijvoorbeeld de correlatie tussen breedtegraad en hoogte in dit onderzoek (R=0,72), maakte het onmogelijk om gevonden variatie aan één van deze twee factoren toe te schrijven. Conclusie was dat in het algemeen de extrapolatie van gevonden verbanden met grote voorzichtigheid moet worden toegepast. De test of het gevoerde management invloed heeft op het fenotype van de planten, wees erop dat begrazing leidt tot de vorming van meer aartjes per aar. Dit zou kunnen dienen als compensatie voor de verminderde generatieve groei (minder aarvorming) die in het algemeen optreedt bij weidetypen.

Binnen de Nederlandse locaties werden steeds drie populaties verzameld, om te analyseren of variaties binnen een locatie kunnen leiden tot fenotypische verschillen tussen populaties. Dit zou van groot belang kunnen zijn voor *in situ* conservering van Engels raaigras populaties. Het bleek dat het soort management de enige factor was, die aantoonbaar leidde tot fenotypisch verschillende populaties binnen een locatie.

In hoofdstuk 6 wordt de allozymvariatie voor dezelfde populaties als in hoofdstuk 5 beschreven. Ook met deze gegevens werd gezocht naar koppelingen met omgevings- en klimaatsfactoren. Daarnaast werd de koppeling met fenotype bepaald. Resultaten wezen uit dat er nauwelijks verbanden bestaan tussen allelen, hun frequenties en omgevings en klimaatsfactoren. De weinige allelen waarvoor wel een verband bestond waren Acp-a, Sdh-b en Sdh-c. Acp-a en Sdh-c kwamen significant meer voor in populaties van meer noordelijke breedtegraad, Sdh-b kwam juist minder voor in deze populaties. Alle allelen konden in bijna iedere populatie gevonden worden, populaties verschilden voornamelijk van elkaar met betrekking tot allel frequenties. Bovendien werd duidelijk dat de cultivars totaal geen reductie in genetische diversiteit vertoonden ten opzichte van verzamelde populaties. De conclusie was dan ook dat voor Engels raaigras allozymdata niet bruikbaar zijn als selectiecriterium voor genenbank accessies.

Ook met behulp van de allozymdata werd bekeken of populaties verzameld binnen de Nederlandse locaties van elkaar verschilden. De resultaten stemden overeen met die van hoofdstuk 5. De meest heterogene locaties met betrekking tot fenotypische variatie vertoonden ook de meeste significante verschillen qua allozymsamenstelling.

De resultaten uit hoofdstuk 5 en 6 tonen aan dat *in situ* conservatie van Engels raaigras tot de mogelijkheden behoort. Op morfologische kenmerken vertonen de verzamelde populaties duidelijke verschillen van de cultivars, bovendien valt er geen variatiereductie waar te nemen. Wel is duidelijk dat locaties voor *in situ* conservatie over geheel Europa verspreid moeten liggen. Breedtegraad, temperatuursfactoren, neerslag, bodemsoort en management kunnen als leidraad dienen voor de keuze van locaties, hoewel niet alle gevonden variatie hiermee verklaard kan worden. De resultaten van de allozym analyses geven aan dat er geen gevaar is voor verlies aan genetische variatie op dit niveau, in geval van *in situ* conservatie.

NAWOORD

Na het lezen van dit proefschrift zal het duidelijk zijn dat er naast mijzelf nog tal van anderen zijn geweest die zich ingezet hebben om dit onderzoek tot een goed einde te brengen. Een aantal van hen wil ik graag op deze plaats persoonlijk bedanken voor al zijn of haar inspanningen.

Allereerst mijn promotor Prof. Dr. Ir. L.J.G. van der Maesen voor het geven van de mogelijkheid tot uitvoeren van dit onderzoek. Als DLO-aio ben ik hiervoor niet alleen de LUW dank verschuldigd maar ook het CPRO-DLO. Met name de afdeling Cultivarstrategie en het Centrum voor Genetische Bronnen. Willem Brandenburg zorgde ervoor dat ik mijn werkplek kreeg, eerst in het tuinhuis en later op het hoofdgebouw van Nergena, en dat ik meedraaide binnen de afdeling Cultivarstrategie. Prof. van der Maesen, Willem Brandenburg, Ronald van den Berg en Theo van Hintum bedank ik voor het vele leeswerk dat zij hebben verricht, en het bruikbare commentaar dat zij gaven op mijn teksten.

Een onmisbare rol speelden Gerda Sabatino-Maasen en Gerda Uenk-Stunnenberg. In weer en wind hebben wij gezamenlijk ontelbare graspollen gemeten op het veld en in het lab. Altijd stipt op tijd en goed gehumeurd, zodat deze klus ieder jaar weer met veel plezier werd geklaard. Ook zorgde zij ervoor dat al het gebruikte plantmateriaal in het herbarium terecht kwam.

Op de Vakgroep Systematiek, Evolutie en Paleobiologie van de Universiteit van Amsterdam werden mij de beginselen van de zetmeel electroforese bijgebracht, Hans den Nijs en Prof. K. Bachman bedank ik voor hun gastvrijheid. Jacquelien Donkers en Dirk Visser voerden vervolgens het grootste gedeelte van de electroforese uit, geen geringe klus! Alle twee met zoveel inzet, dat ik er nauwelijks omkijken naar had.

Jan van Hardeveld en Gerrit van der Wardt waren behulpzaam met het verzamelen van de Nederlandse populaties. Ook maakten zij, samen met hun medewerk(st)ers, de pauzes in de landbouwkas altijd een gezellige boel. De proefvelddienst verzorgde de opkweek, het uitplanten en de verzorging van tal van grasplanten, waardoor de waarnemingen altijd vlekkeloos konden verlopen. De sectie landbouw van het RKO en met name Dick Klein-Geltink en Nienhuis, zorgden ervoor dat in het begin van dit onderzoek ik snel thuis was in de grassen. Dick ging zelfs zo ver dat hij tijdens zijn vakantie een populatie verzamelde om te gebruiken in het onderzoek (zie Hoofdstuk 5 en 6).

Het Gelderse, Drentse, Overijssels en het Zeeuws Landschap ben ik zeer erkentelijk voor het feit dat zij het mogelijk maakte om populaties raaigras in hun terreinen te verzamelen, een essentieel onderdeel van dit onderzoek.

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Diverse personen en instanties leverden illustratie materiaal voor dit proefschrift. Allereerst Henny Ansink die met veel geduld *Lolium* zaden op spelden wist te prikken, en de foto's uit hoofdstuk 4 maakte. Het Uppsala herbarium leverde de foto van het type exemplaar van *L. temulentum*, en het Jan Kops huis zorgde voor de foto's uit de *Pinax* van Bauhin.

Jolanda Piket deed tijdens haar afstudeervak plantentaxonomie de metingen van zaadkenmerken en de electroforese van zaadeiwitten. Jos van Vooren, Gerie van der Heijden en Gerrit Polder, kortom de sectie Beeldanalyse, zorgden ervoor dat het mogelijk was om alle *Lolium* zaden met behulp van beeldanalyse te meten. Vooral Jos was altijd bereid om 'even' te helpen.

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En tot slot alle Nergenezen en Binnenhavers die hier niet met name genoemd staan, maar die ervoor gezorgd hebben dat mijn tijd op het RIVRO→CRZ→CPRO meer dan prettig is verlopen!

Birgit

CURRICULUM VITAE

Birgitta Pamela Loos werd geboren op 5 juni 1966 te Valkenswaard. Na het behalen van het diploma Atheneum-B aan het Hertog Jan College in Valkenswaard, studeerde zij vanaf september 1984 Plantenveredeling aan de Landbouwuniversiteit Wageningen. Haar stage werd doorgebracht op het Cereal Rust Laboratory in St. Paul, Minnesota (USA), van mei tot en met september 1988. In november 1989 studeerde zij af met als hoofdvakken Plantenveredeling en Fytopathologie, en als bijvak Plantentaxonomie. Vanaf 1 januari 1990 was zij werkzaam als assistent-in-opleiding bij de vakgroep Plantentaxonomie van de Landbouwuniversiteit, maar gedetacheerd op het Centrum voor Plantenveredelings- en Reproductie Onderzoek (CPRO-DLO). Vanaf 1 december 1993 is zij als milieuhygiënisch medewerkster werkzaam bij het Milieu Adviesbureau Mebo, op het gebied van de kennisgeving van genetisch gemodificeerde organismen.