Molecular markers in management of *ex situ* PGR – A case study

ANDREAS BÖRNER[1,*], ELENA K KHLESTKINA[2], SABINA CHEBOTAR[3], MANUELA NAGEL[1], MIAN ABDUR REHMAN ARIF[1], KERSTIN NEUMANN[1], BORISLAV KOBILJSKI[4], ULRIKE LOHWASSER[1] and MARION S RÖDER[1]

[1]*Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstrasse 3 06466 Gatersleben, Germany*

[2]*Institute of Cytology and Genetics (ICG), Siberian Branch of the Russian Academy of Sciences, Lavrentjeva Ave. 10, Novosibirsk 630090, Russian Federation*

[3]*South Plant Biotechnology Center, Ovidiopolskaya dor. 3, 65036 Odessa, Ukraine*

[4]*Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia*

**Corresponding author (Fax, +49-39482-5155; Email, boerner@ipk-gatersleben.de)*

Worldwide germplasm collections contain about 7.4 million accessions of plant genetic resources for food and agriculture. One of the 10 largest *ex situ* genebanks of our globe is located at the Leibniz Institute of Plant Genetics and Crop Plant Research in Gatersleben, Germany. Molecular tools have been used for various gene bank management practices including characterization and utilization of the germplasm. The results on genetic integrity of long-term-stored gene bank accessions of wheat (self-pollinating) and rye (open-pollinating) cereal crops revealed a high degree of identity for wheat. In contrast, the out-pollinating accessions of rye exhibited shifts in allele frequencies. The genetic diversity of wheat and barley germplasm collected at intervals of 40 to 50 years in comparable geographical regions showed qualitative rather than a quantitative change in diversity. The inter- and intraspecific variation of seed longevity was analysed and differences were detected. Genetic studies in barley, wheat and oilseed rape revealed numerous QTL, indicating the complex and quantitative nature of seed longevity. Some of the loci identified were in genomic regions that co-localize with genes determining agronomic traits such as spike architecture or biotic and abiotic stress response. Finally, a genome-wide association mapping analysis of a core collection of wheat for flowering time was performed using diversity array technology (DArT) markers. Maker trait associations were detected in genomic regions where major genes or QTL have been described earlier. In addition, new loci were also detected, providing opportunities to monitor genetic variation for crop improvement.

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1. Introduction

Plant genetic resources for food and agriculture (PGRFA) play a major role for global food security. The most significant and widespread approach of conserving PGRFA is *ex situ* conservation. Worldwide 7.4 million accessions are conserved in about 1,500 *ex situ* genebanks. The 10 largest collections are listed in table 1 (FAO 2010).

With a total inventory of 150,000 accessions of 3,212 plant species and 776 genera, the 'Federal *Ex situ* Genebank of Germany' in Gatersleben holds one of the most comprehensive collections worldwide. It comprises wild and primitive forms, landraces as well as old and more recent cultivars of mainly cereals but also other crops (figure 1). Starting in the 1920s material was accumulated systematically.

Seed storage is being managed in large cold chambers at −18°C. Seeds are kept in glass jars, covered with bags containing silica gel. The maintenance of the collection requires periodical regeneration. Each year between 8% and 10% of the collection is grown either in the field or in

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Table 1. The 10 largest germplasm collections on earth

Institution	Country	Accessions
NPGS (National Plant Germplasm System)	USA	508,994
ICGR-CAAS (Institute of Crop Germplasm Resources, Chinese Academy of Agricultural Science)	China	391,919
NBPGR (National Bureau of Plant Genetic Resources)	India	366,333
VIR (N. I. Vavilov Research Institute of Plant Industry)	Russia	322,238
NIAS (National Institute of Agrobiological Science)	Japan	243,463
CIMMYT (Centro Internacional de Mejoramiento de Maíz y Trigo)	Mexico	173,571
IPK (Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung)	Germany	151,408
ICARDA (International Center for Agricultural Research in the Dry Areas)	Syria	132,793
ICRISAT (International Crops Research Institute for the Semi-Arid Tropics)	India	118,882
IRRI (International Rice Research Institute)	Philippines	109,161

the glasshouse. Regeneration becomes necessary when: (1) the quantity of stored seed has dropped below a pre-set threshold, due to supply to users, (2) viability falls below a pre-set threshold, (3) phenotypic evaluations of the accessions need to be conducted or (4) new accessions, which require multiplication and characterization, enter the collection. Regeneration is carried out locally to ensure genetic integrity and to minimize genetic erosion. Voucher specimens, photographs and written documentation are used to monitor the identity of the material. Special attention has to be given to out-pollinating species, which are either multiplied in a small glasshouse or in isolation plots in the field.

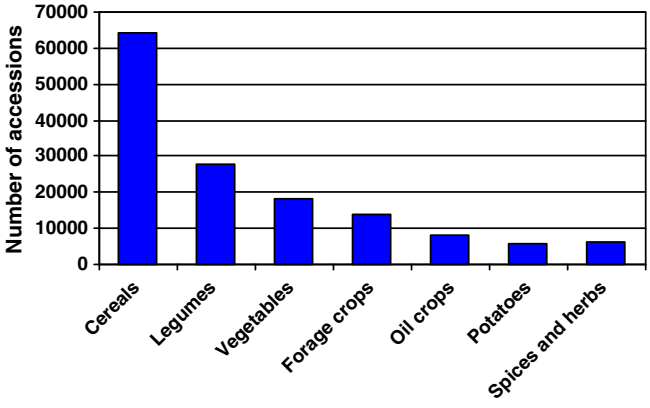

Figure 1. Inventory of the Gatersleben *ex situ* collection.

Major research areas include genetic integrity, genetic diversity and seed longevity. An extensive programme of germplasm evaluation and genetic characterisation was undertaken. Results on the successful utilization of molecular tools for germplasm analysis are presented.

2. Genetic integrity of long-term-stored genebank accessions

A particular challenge for *ex situ* genebanks is the maintenance of genetic integrity. Outcrossing or inadequate handling during multiplication will inevitably downgrade genetic identity. Molecular markers (microsatellites) were used at IPK to quantify and minimize this potential problem by maintaining both the seed harvested from the most recent regeneration and a herbarium voucher specimen collected from the initial multiplication. The cereal herbarium collection includes samples of both the grain and intact spikes of each accession.

The fingerprinting of wheat accessions (five grains per sample), maintained in the Gatersleben genebank and regenerated up to 24 times over a 50 year period, revealed a high degree of identity (figure 2). No contamination due to foreign pollen or incorrect handling during the multiplication cycles was discovered, indicating the precautions taken by the IPK staff to preserve the genetic integrity (Börner *et al.* 2000).

A similar analysis of out-pollinating rye accessions, regenerated 2–13 times, was performed analysing 36 grains of each accession derived from the herbarium collection (number of seeds was limited) and 60 grains from the most recent regeneration cycle. Here extensive shifts in allele frequencies were detected. Whereas at some loci a decreased frequency or even loss of alleles was observed, at others new alleles were detected, suggesting outcrossing with other populations. The extent of changes observed was related to the number of multiplication cycles (figure 3). These results suggest the need to review the regeneration management for out-pollinating species in *ex situ* collections, specifically related to the optimisation of population size and distance between regeneration plots (Chebotar *et al.* 2003).

3. Genetic diversity of wheat and barley germplasm

Human activities, in particular urbanization, the replacement of traditional agricultural systems by modern industrial methods and the introduction of modern high-yielding varieties, may pose a danger to biological diversity. Plant specialists from several countries, including Germany, initiated collection missions in the early 1920s to accumulate and store genetic resources *ex situ*. Fresh collections from certain

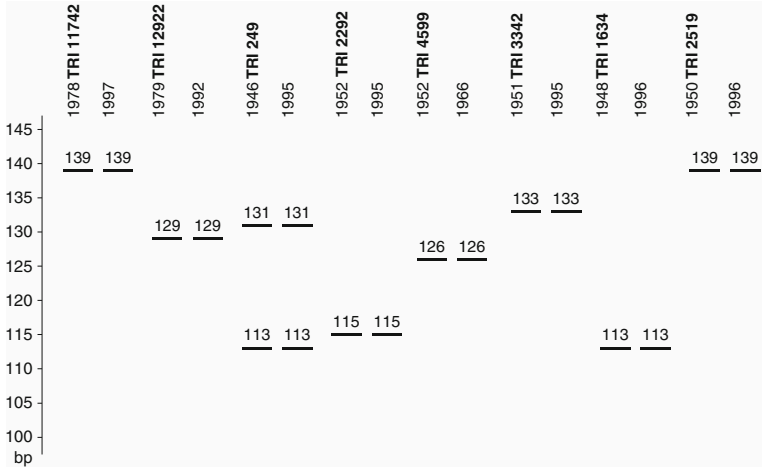

Figure 2. Microsatellites amplified from the DNA extracted from grain harvested from the original (on the left) and most recent (on the right) multiplication of eight bread wheat accessions, showing the maintenance of genetic integrity after long-term storage. TRI (= *Triticum*) is used as abbreviation for ID numbers in the Gatersleben genebank.

areas were carried out after the passage of 40 to 50 years in Austria, Nepal, North India and Albania. The distribution of the collection sites within the countries was highly similar (figure 4), and therefore the material provides an excellent basis to quantify changes in genetic diversity over time. A case study for wheat has been reported by Khlestkina *et al.* (2004).

Genetic diversity was assessed on the basis of variation at selected microsatellite loci distributed randomly across the genome. The mean number of alleles and the polymorphic information content (PIC) at each locus were calculated. There was an overall stability in the extent of genetic diversity over the 40 to 50 year collection period in all the geographical regions investigated. However, there was evidence for qualitative changes in diversity, as one-third of the alleles detected were unique to a single collection period (figure 5). These findings demonstrate that an allele flow (qualitative change) took place during the evolution of traditional agriculture to modern production systems, whereas the quantitative level of genetic diversity was rather stable. It was concluded that in *ex situ* collections one can only preserve the existing allelic composition which may change over time in nature.

In barley a comparable study based on genomic (g-SSR) and EST-derived (e-SSR) microsatellite markers uniformly distributed across the genome was conducted in three comparable geographical regions in Austria, Albania and India (Khlestkina *et al.* 2006). The analysis also indicated an absence of any significant differences either in the total number of alleles per locus or in PIC values from the Indian and Austrian materials. However, a slight reduction in genetic diversity was noted among the materials collected in Albania. As for wheat, the trend in numbers of collection mission-specific SSR alleles detected in the early or late expeditions only suggests significant allele flow over the time interval (40 to 50 years) sampled. The g-SSRs showed a higher mean number of alleles/locus and a superior PIC than the e-SSR markers. Again it was concluded that a qualitative rather than a quantitative shift in diversity has taken place over time, and that g-SSR markers detected more diversity than do e-SSR markers.

It should be mentioned here that a range of other studies examining the genetic diversity of wheat (Donini *et al.* 2000; Manifesto *et al.* 2001; Christiansen *et al.* 2002; Roussel *et al.* 2004) and barley (Backes *et al.* 2003; Koebner *et al.* 2003) cultivars had also revealed little evidence that a quantitative diversity loss had resulted from breeding activity. The authors compared old and modern cultivars released in different countries.

4. Inter- and intraspecific variation of Seed longevity

Since the majority of genebank accessions globally are stored as seed, seed longevity is of particular importance for crop germplasm conservation. At the IPK, research was initiated for a range of crops stored in the genebank over decades. Variation between crop species was detected for seeds stored at around 20°C and 50% relative humidity (Nagel and Börner 2010). From 18 species under investigation, pea (*Pisum sativum*) and chive (*Allium schoenoprasum*) seeds retained the viability over the longest (29 years) and shortest (5 years) periods, respectively.

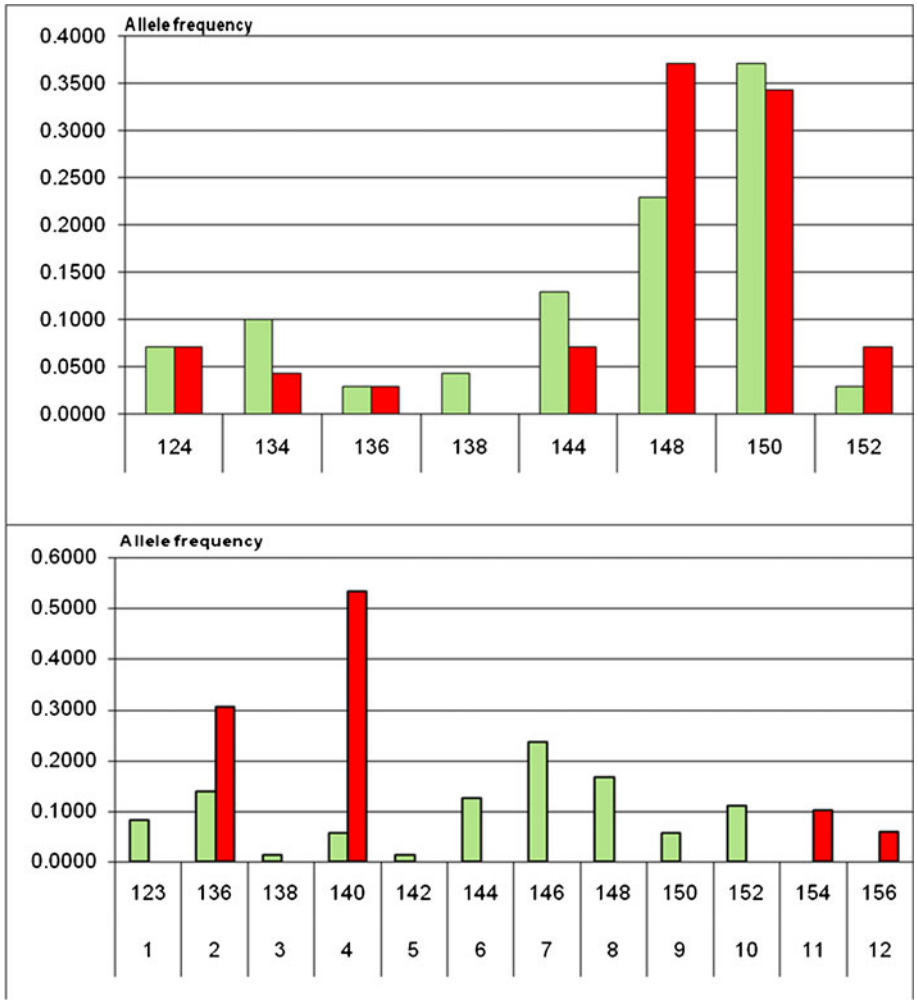

Figure 3. Variation in allele frequency originated from the first (green columns) and most recent (red columns) regeneration detected for SSR marker RMS18 in grain samples of rye accession R784 (above; 2 cycles) and R 78 (below; 12 cycles).

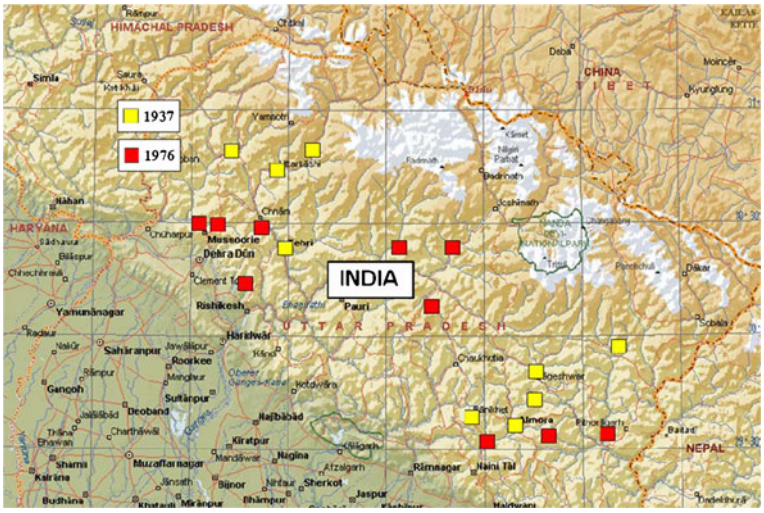

Figure 4. Collection sites of repeat collection expeditions to India in 1937 (yellow boxes) and 1976 (red boxes).

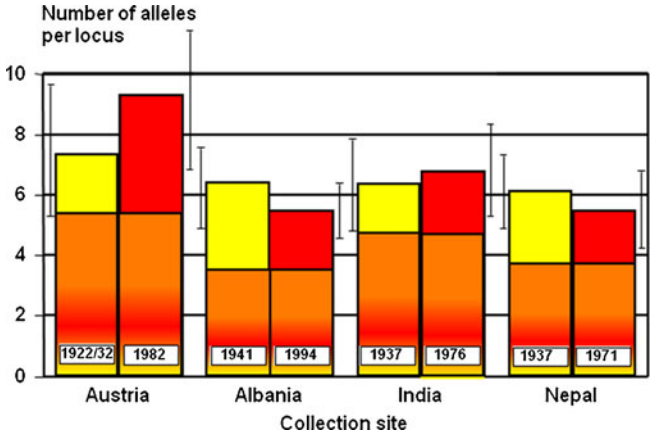

Figure 5. Mean number of alleles per SSR locus detected in bread wheat accessions collected during expeditions to Austria (1922/32 and 1982), Albania (1941 and 1994), India (1937 and 1976) and Nepal (1937 and 1971). Unique alleles are shown in yellow (old expeditions) or red (fresh expeditions) columns, common alleles are indicated by a colour mix.

Intraspecific variation of genebank collections stored for 26 to 33 years at 0°C was investigated by Nagel *et al.* (2010). Crops showed high germination when germinated within 5 years post harvest, but germination of most accessions within species separated strongly after 20 years. In particular, wheat germination varied between 0 and 87% after 34 years of storage, whereas barley accessions had germination between 43% and 95% after 35 years (figure 6). Because the accessions within the species under investigation were regenerated and harvested at the same place in the same year, the same harvest (threshing and cleaning) technologies were applied and the storage conditions were identical, it can be concluded that there are genetic differences in germination after long-term storage.

Therefore, genetic analyses of seed longevity were initiated using accelerated ageing tests based on International Seed Testing Association (ISTA) protocols (ISTA 2008). QTL mapping was performed for barley (Nagel *et al.* 2009), wheat (Rehman Arif *et al.* 2012) and oilseed rape (Nagel *et al.* 2011). Loci detected for seed longevity in barley and wheat mapped to regions already associated with the determination of traits like plant height, naked/hulled grains and spike density just as abiotic and biotic stress response (figure 7).

5. Genome-wide association mapping analysis in wheat

A core collection of 96 winter wheat genotypes from 21 different countries and five continents was selected for a genome-wide association mapping analysis. These genotypes were extracted from a collection of 2,500 genotypes maintained at the Institute of Field and Vegetable Crops, Novi Sad, Serbia, and based on contrasting phenotypic expression for breeding traits (Kobiljski *et al.* 2002). The collection was phenotyped for flowering time in field plots in Novi Sad during six growing seasons between 1994 and 1999. Genotyping using DArT markers was performed by Triticarte Pty. Ltd. (Canberra, Australia; *http://www.triticarte.com.au/*). In total 874 polymorphic markers were used. The associations between markers and traits were calculated with the software programme TASSEL 2.01 (Bradbury *et al.* 2007). The general linear model (GLM) with the Q-Matrix from STRUCTURE as correction for population structure was used. In addition, we applied the version TASSEL 2.1 exploiting the mixed linear model (MLM) using Q-Matrix and the kinship-Matrix (Yu *et al.* 2006). Marker trait associations (MTAs) significant in both models and with $p<0.05$ in 4 out of 6 years were taken into account only.

Details for genetic map, population structure and linkage disequilibrium of the given core collection are described by Neumann *et al.* (2011). For flowering time, 13 MTAs were detected (figure 8) on chromosomes 1B, 1D (2 markers), 2B, 2D, 4B, 5B, 5D, 6A (2 markers), 6B and 7A (2 closely linked markers). The MTA on the short arm of chromosome 2D may represent a major photoperiod response (*Ppd*) gene, whereas the associations in the centromere regions of chromosomes 1B and 1D may reflect variation at known earliness *per se* (*Eps*) genes (McIntosh *et al.* 2008). The closely linked MTAs on chromosome 7A may be orthologous to a QTL for photoperiod response detected by Khlestkina *et al.*

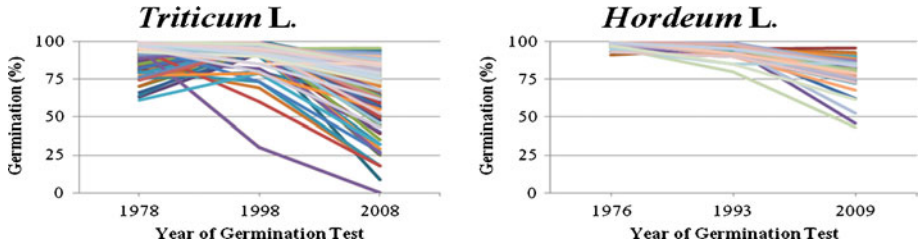

Figure 6. Mean germination of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) accessions over various years of testing.

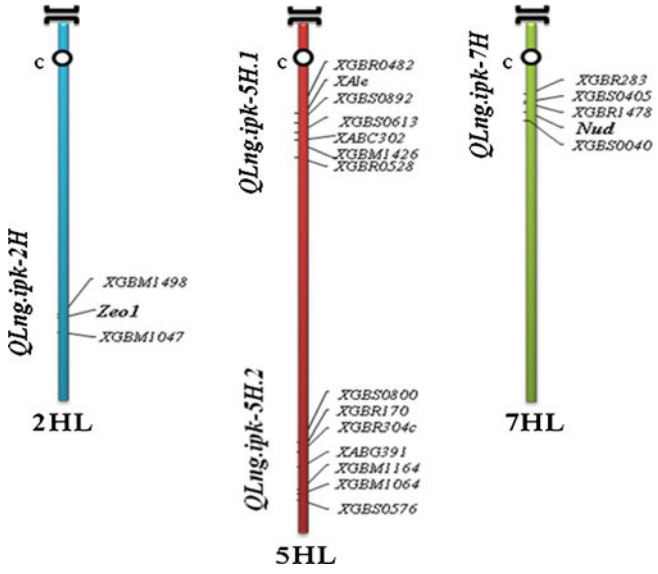

Figure 7. Major QTLs for seed longevity (*QLng*) detected on barley chromosomes 2HL, 5HL and 7HL.

(2009) in a homologous region on chromosome 7B. The remaining loci were detected for the first time but need further validation.

6. Conclusions

- A large collection is lying unutilized in global *ex situ* collections.
- The genetic integrity of self-pollinators is maintained while the open-pollinators changes during regeneration cycles.
- There is little evidence for a significant erosion of overall diversity. A qualitative rather than a quantitative shift appeared.

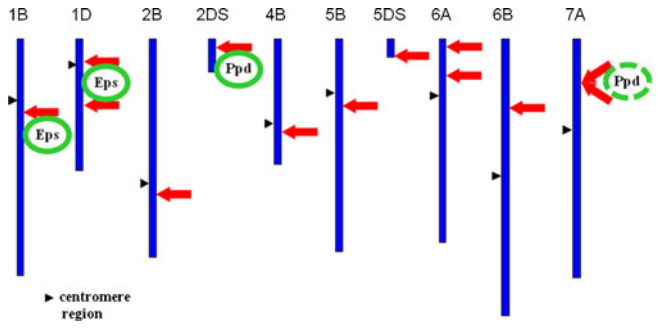

Figure 8. MTAs for flowering time marked by red arrows. Positions of similar genes/QTLs described earlier are indicated below arrows (ellipses).

- Longevity of the germplasm in seed banks remains for a limited time in storage. There is need for research on genetics and physiology of seed longevity.
- Association mapping analysis is useful for detection of new genes/alleles.

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