1. General Introduction

The Need for new Plant Varieties

Since the 1960s, the human world population has more than doubled from 3 billion to currently more than 6 billion and it is estimated to reach 8 billion by the year 2025 (Vaupel et al., 1998). At the same time, the amount of arable land is decreasing due to diversion to nonfarm uses, such as urban or industrial development or natural phenomena such as expanding deserts. Therefore, the only option is to produce more food on less land to meet the needs of the increasing population. Thus, the critical importance of ecologically sustainable advances in the productivity and profitability of major farming systems is evident. At present, the average yield increase of major crops such as maize (*Zea mays* L.) (Duvick and Cassman, 1999), rice (*Oryza sativa* L.) (Mann, 1997; Vasil, 1998), soybean (*Glycine max* L.) (Ustun et al., 2001) or wheat (*Triticum aestivum* L.) (Reynolds et al., 1999) amounts to approx. 0.5 - 1.5% per year. This yield increase is the result of improvements in soil cultivation, fertilization, and plant protection, but also to a large extent by continuous breeding progress made by plant breeders for all crops, because 50% of the yearly yield increase in crop production is attributed to genetic modification and selection. Therefore, plant breeding is playing a key role in this process, because the average yield increase of major crops is currently slightly lower than the growth rate of the world population of currently approx. 1.8% *p.a.* (Daily et al., 1998). Consequently, the particular importance of the continuous development of new plant varieties in all major crops is obvious to contribute to improvements in quantity, quality, and cost of satisfying the requirements for food, fuel, fiber and for raw materials for industry (Tanksley and McCouch, 1997).

The Need for legal Protection of new Plant Varieties

Plant breeding aims at modifying plants genetically in a way to adapt them better to the specified needs of the people (Becker, 1993) and requires extensive crossing programs and sophisticated selection strategies. Thus, procedures of breeding new crop varieties are time-consuming and expensive, *e.g.,* the development of a new maize hybrid takes up to 15

years and requires monetary investments of up to 5.000.000 US$ (Troyer et al., 2002). Expenditures of more than one billion US$ were achieved in 2000 only by US plant breeding companies, combined with a research and development quota (R&D-quota) of approx. 15%, which makes plant breeding one of the most research intensive business fields (Heisey et al., 1999).

Biological organisms including plants are characterized by the ability to reproduce or replicate itself. This attribute faces plant breeders with special problems since the release of propagating material of variety to growers enables them in many cases to reproduce the variety without further recourse to the breeder. In addition, the breeder's competitors can secure supplies of propagating material and compete in a short time profiting from the breeding effort of the breeder of the original variety over many years. In order to secure an appropriate reward for the investments made, it is important to protect plant varieties in the best possible way against plagiarism and misuse as intellectual properties of the breeders (International Seed Federation, 2002). This protection can either be warranted by requesting patent protection for new plant varieties, as is possible *e.g.* in the USA, or by applying for plant varietal protection (PVP).

Plant varietal Protection within the UPOV-System

Besides patents, many countries have established systems whereby exclusive rights of exploitation are granted to the breeders of new varieties in order to provide breeders with an opportunity to receive a reasonable return on past investments. Furthermore, PVP systems were implemented to provide an incentive for continued or increased investment in the future and to recognize the moral right of the innovator to be recognized as such and his economic right to receive remuneration for his efforts.

The International Union for the Protection of New Varieties of Plants (UPOV) is a union of states which have agreed to grant exclusive rights of exploitation to the breeders of new plant varieties on an internationally harmonized basis. Currently, 52 countries joined the UPOV convention (UPOV, 1961) and implemented their legal regulations into their national PVP acts. Accordingly, a variety needs to be distinguishable from all other varieties of the same crop, uniform, new, and stable to receive the privilege of PVP, as

assessed with the testing procedures for distinctness, uniformity and stability (DUS). These well-defined procedures for DUS-testing relying on morphological and biochemical traits are the basis of PVP for numerous crops.

The "Breeders' Exemption"

Apart from the protection against misuse, however, a variety needs to be freely available to all breeders as a germplasm resource to secure sustainable breeding progress. Therefore, the principle of the "breeder's exemption" was implemented into the 1978 act of the UPOV convention (UPOV, 1978), enabling breeders to use protected varieties for the purpose of producing new varieties. Accordingly, any protected variety may be freely used as a source of initial variation in breeding programs to develop further varieties and any such variety may itself be protected and exploited without any obligation on the part of its breeder.

For the purpose of breeding new varieties, the breeder's exemption is a fundamental aspect of the UPOV system of PVP and represents the main difference between PVP and patents. It warrants a continuous breeding progress on the basis of the PVP system and recognizes that real progress in breeding, which for the benefit of society must be the goal of intellectual property rights in this field, relies on access to the latest improvements and new variation. Therefore, access is warranted to all breeding materials in the form of modern varieties, as well as landraces and wild species, to achieve the greatest progress and is only possible if protected varieties are available for further breeding.

Misuse of the Breeders' Exemption

Recently, newly developed methods and strategies have created the technical basis for a misuse of the breeder's exemption. Among others, these methods comprise repeated (marker-assisted) backcrossing to protected varieties, the addition of one or few genes to protected varieties by genetic engineering, reselection within protected varieties (*e.g.,* selection for natural or induced mutants within ornamental crops) and the generation of "cosmetic" variation within a protected variety, (*e.g.*, generation of somaclonal variants by repeated tissue culture cycles or selection of clones within synthetics) (Knaak et al., 1996).

These techniques provide the possibility to undermine the PVP system in the original intention of the UPOV convention, which implied to support classical, creative plant breeding. Therefore, it has become possible to plagiarize protected varieties without indemnification for the breeders of the corresponding original varieties and very little genetic variation is created.

Essentially Derived Varieties

The concept of essentially derived varieties (EDVs) was implemented into the 1991 act of the UPOV convention to prevent plagiarized breeding and to allow at the same time the use of protected varieties for the development of new cultivars. A variety is deemed to be essentially derived from an initial variety (IV) when it is (i) predominantly derived from the IV, or from a variety that is itself predominantly derived from the IV, while retaining the expression of the essential characteristics that result from the genotype or combination of genotypes of the initial variety, (ii) it is clearly distinguishable from the initial variety and (iii) except for differences, which result from the act of derivation, it conforms to the initial variety in the expression of essential characteristics that result from the genotype or combination of genotypes of the initial variety (UPOV, 1991).
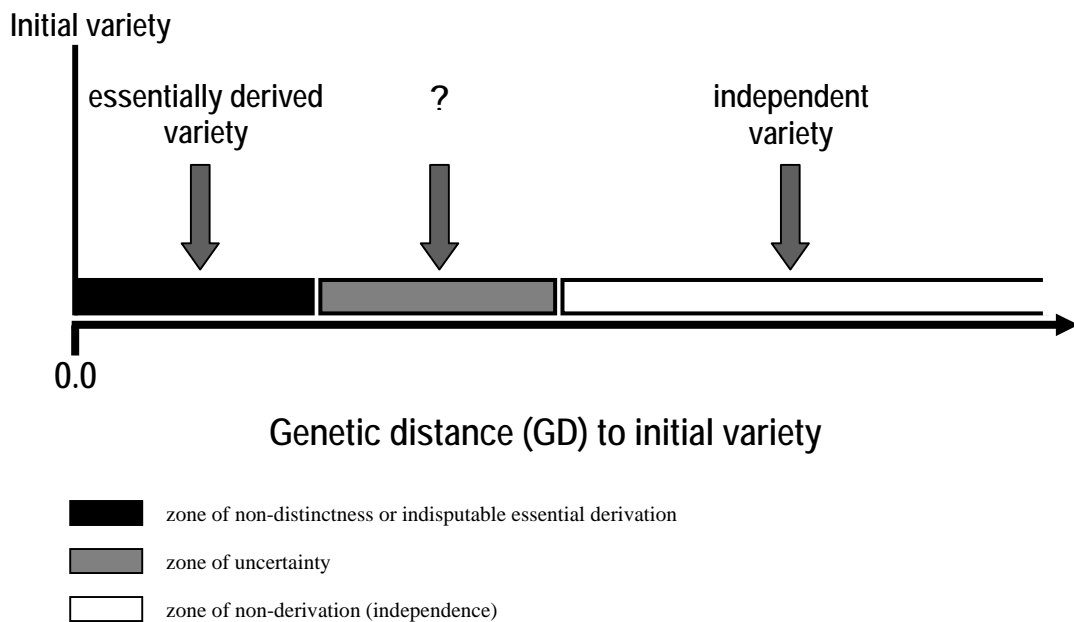

Figure 1: The concept of different thresholds to discriminate between essentially derived and independent varieties based on their GD according to an initial variety.

According to ASSINSEL, (1999a), a worldwide association of plant breeders, a variety is essentially derived if all three criteria are met. If one of the criteria is not fulfilled, there is no essential derivation. In contrast to DUS testing for PVP, which is performed by governmental organizations, the breeder of the IV is supposed to supply evidence that a new variety was essentially derived from the IV. To prove a predominant derivation, either a directly documented evidence will be necessary (Gilliland et al., 2000), or forensic approaches will have to be applied, similar to those applied in human genetics for detecting parentage (Gill et al., 1995; Graham et al., 2000). Distinctness will most likely be observed by DUS-testing, however, no consensus has currently been achieved regarding the methods to be used for determining the expression of the essential characteristics and to observe the genetic conformity between IVs and EDVs.

In addition, the breeders have not yet agreed on accepted or non-accepted breeding procedures yielding independently derived varieties (IDVs) or EDVs, respectively. In particular, the number of acceptable backcrosses to a protected variety without generating an EDV has not been fixed for all major crops including maize. Therefore, appropriate methods to identify EDVs have not been defined, because a detailed comparison of all eligible identification methods has not been performed so far. Theoretically, genetic distances (GDs) based on molecular markers, morphological traits as used for DUS testing, probabilistic values such as the coancestry coefficient (Malécot, 1948), or other descriptors like heterosis or combining ability could serve for identification of EDVs because they all provide information on the relatedness of cultivars (International Seed Federation, 2002). Due to this lack of knowledge, thresholds to distinguish between IVs and EDVs have not yet been defined (Fig. 1.). Thus, the EDV concept was implemented into practical plant breeding only for very few crops, such as lettuce (International Seed Federation, 2003), but is still not used for all major crops, including maize.

Identification of EDVs

As the concept of essential derivation is rather based on genotypes than on phenotypes, the use of scientifically reliable methods to observe GDs is required by breeding organizations, such as ASSINSEL, (1999b). Therefore, justiciable criteria have to be de-

veloped and validated because of the legal consequences for infringing PVP granted within the UPOV-system.

Genetic fingerprints based on molecular marker data proved to be reliable tools to determine parentage and to identify crime suspects in humans (Gill et al., 1995; Graham et al., 2000). Like in criminology, molecular markers became key instruments in the plant breeding sector to evaluate the illegal use of germplasm (Martin et al., 1991). Therefore, molecular markers were proposed repeatedly as suitable tools to identify essential derivation in all major crops (ASSINSEL, 1999a; Bernardo and Kahler, 2001; Knaak et al., 1996; Roldan Ruiz et al., 2000; Smith and Smith, 1989b), as they provide a direct measure of the true relatedness of two cultivars.

In barley (*Hordeum vulgare* L.), restriction fragment length polymorphisms (RFLPs) (Graner et al., 1994) as well as amplified fragment length polymorphisms (AFLPs) (Schut et al., 1997) or simple sequence repeats (SSRs) (Russell et al., 1997) were successfully used to separate spring from winter cultivars. In wheat (*Triticum aestivum* L.) RFLP and SSR markers were applied to divide germplasm into high and low quality types (Bohn et al., 1999) or to evaluate close pedigree relationships (Plaschke et al., 1995). In addition, all studies yielded significant correlations between the coancestry coefficient (*f*) (Malécot, 1948) and GDs and were consequently useful for revealing pedigree relationships among cultivars.

As shown in all other major crops, molecular markers proved to be an important tool for studying genetic relationships of inbred lines within and between gene pools in maize (*Zea mays* L.). GDs based on molecular marker data were used to evaluate genetic diversity and to divide germplasm into gene pools. It was shown that GD values revealed by RFLPs (Messmer et al., 1993) as well as random amplified polymorphic DNA (RAPDs) (Hahn et al., 1995), AFLPs (Lübberstedt et al., 2000) or SSRs (Smith et al., 1997) are adequate estimates for the underlying pedigree relationships. Furthermore, Lübberstedt et al. (2000) and Pejic et al. (1998) demonstrated that GDs revealed by different marker systems were significantly correlated among each other and with *f*.

Hypothetically, *f* itself could be used for identification of EDVs, because it yields an estimator of the relationship between two genotypes and reflects the breeding procedure applied to derive a potential EDV from an IV. However, use of *f* is associated with several

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major drawbacks, as it is only the expected value of the genetic similarity (1-GD) of two individuals and provides no information on the true genetic relatedness (Messmer et al., 1993). In addition, *f* is based on assumptions that are mostly unrealistic (Melchinger et al., 1991), *e.g.*, all lines in the pedigree pathway are homogeneous and homozygous, lines with no common parentage have *f*=0, or lines derived from a cross obtained half of the genome from each parent. Moreover, reliable pedigree data will most likely be unavailable in case of a suspected EDV.

The estimation of the conformity of the essential characteristics by the use of phenotypic data, such as morphological traits or heterosis, has been proposed and is still under debate (ASSINSEL, 1999a). Hitherto, accurate morphological and agronomic descriptions of cultivars and varieties are the basis of world-wide PVP systems and assure farmers and breeders that they are using clearly identifiable varieties to high standards of purity and quality (Smith and Smith, 1989a). In addition, numerous studies showed significant correlations between midparent heterosis and the coefficient of parentage (*f*) (Melchinger, 1999; Smith et al., 1991). For these reasons, proponents of morphological traits or heterosis for identifications of EDVs argue that phenotypic information provides the basis for PVP and should, thus, also be used for identification of EDVs.

Critical data on the scientific reliability for all kinds of distance measures, as requested by ASSINSEL (1999b) is still lacking. Potential reproducibility problems of molecular marker data were investigated by Jones et al. (1997), who reported scoring differences of up to 2 base pairs (bp) among the same SSR fragments detected by different laboratories. In sugar beet (*Beta vulgaris L.*) and wild *Beta* species, a reproducibility of AFLP bands of 97.6 % was determined by performing all necessary analytical steps twice (Hansen et al., 1999). In contrast to these results, Jones et al. (1997) and Bagley et al. (2001) reported an extremely high reproducibility of AFLP bands close to 100%. In addition, the relationship between morphological distances and GDs based on molecular markers or the coancestry coefficient (*f*) in maize was observed to be triangular instead of linear (Dillmann and Guérin, 1998). Furthermore, associations among maize inbred lines on the basis of morphology were not correlated with distance measures derived from heterosis or pedigree data (Smith and Smith, 1989b). However, no detailed comparison of GDs based on molecular markers, morphological data, and heterosis on their ability to accurately identify EDVs is available hitherto.

Objectives

The main goal of this study was to examine the usefulness of molecular markers, morphological traits, and heterosis for identifying EDVs in maize. In detail, the objectives of this thesis were to (1) investigate genetic and technical sources of variation in SSR and AFLP data within maize inbred lines and assess their impact on identification of EDVs, (2) analyze factors influencing GDs based on SSRs and AFLPs between related maize inbred lines, (3) investigate the power of SSR- and AFLP-based GD estimates for discriminating between progenies derived from F_2, BC_1, and BC_2 populations, (4) exemplify the theoretical and simulated results of the statistical theory introduced by Bohn et al. (2003) with experimental data, (5) compare the results observed for molecular markers to those obtained with morphological traits and heterosis, and (6) draw conclusions with regard to a detailed comparison of the various distance measures and EDV thresholds suggested in the literature.

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