Qualitative Inheritance Analysis of Isoenzymes in Haploid Gametophytes: Principles and a Computerized Method

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Abstract

Isoenzyme banding patterns show unique and complex inheritance with no counterpart among DNA markers. The objective of inheritance analysis, without which advanced population genetic studies are not possible, is to determine the degree of polymerization and number of loci involved and to identify each isoenzyme band with alleles at gene loci. If enzyme gene loci are active in both diplo- and haplophase tissue, segregation of the isoenzyme bands expressed by a diplophase individual among its gametes facilitates genetic interpretation of the bands. Such tissue can be studied in the haploid primary endosperm (megagametophyte) of conifer seeds and in (doubled) haploid plants. An example demonstrates that even in such ideal cases numerous genetic interpretations may exist.

The conceptual basis of inheritance analysis is developed with particular attention to the fundamental concept of transmission homology within individuals and populations. These concepts are then applied to the inheritance analysis of isoenzyme banding patterns observed in haploid gametophytes. A computer program (HAPLOZYM) is introduced that systematically generates all hypothetical modes of inheritance that conform to certain qualitative rules for the interpretation of single isoenzyme bands.

Key words: isoenzymes, inheritance analysis, haploid, transmission homology, computer program.

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Introduction

In most higher plants, hypotheses about the mode of inheritance of variable traits have traditionally been formulated and tested by observing the phenotypes of diplophase individuals and their sexually produced, diplophase progeny. Controlled self-fertilizations as performed by MENDEL (1866) or controlled crosses, if possible over several generations, still form the definitive method of determining mode of inheritance using diplophase material. If controlled crosses are infeasible, analysis of progeny from open pollination of single individuals in a population can provide an alternative (GILLET and HATTEMER, 1989). All of these methods allow inferences on the mode of inheritance only after fusion of the parental gametes. They are based on the concept that the diplophase phenotypes consistently derive from the genetic information contributed by two haplophases – the gametes.

Tracing diplophase phenotypes back to the underlying haploid genetic information is easier if the haplophase generation – in plants the gametophyte – is directly observable. Probably the most well-studied example in spermatophytes is the primary endosperm (or megagametophyte) of conifer seeds. As the female prothallium, which is formed from the megaspore before gametic fusion as nutritive tissue for the developing embryo, the primary endosperm contains only the haploid genetic information of the megaspore. In almost all coniferous species, the primary endosperm is of a size sufficient for biochemical analysis (*Cupressus sempervirens* L. may be the only exception described to date (PAPAGEORGIOU *et al.* 1993)). Over the past 20 years in an ever increasing number of conifer species, the primary endosperm has been used to infer the genotype of the mother tree and to distinguish the maternal and paternal gametic contributions to the seed embryo at loci controlling isoenzymes, "the electrophoretically separable variants of one enzyme species (or system)" (BERGMANN *et al.,* 1989). The vast literature on the subject is reflected in the various contributions to ADAMS *et al.* (1992).

Additional examples of observable haplophase tissue underline the importance of concepts for haploid analysis: Bryophytes and mosses have green, autotrophic gametophytes. In many spermatophytes, haploid (or completely homozygous doubled haploid) organisms are producible by the induction of andro- or gynogenesis in gametophytes (see CHEN (1987) for review of hardwood trees, ROHR (1987) for gymnosperms, THOMPSON *et al.* (1991) and references therein for crop plants). Additionally, the PCR technique may soon make it possible to analyze the DNA of single pollen grains (B. VORNAM, personal communication).

The utility of gametophytic tissue for inheritance analysis of isoenzymes stems not only from the observation that most of the enzyme systems that are active in the gametophyte are also active in the diploid tissue of the sporophyte. The fact that the same single isoenzymes present in the gametophyte are usually also present in sporophytic tissue of the parent, as evidenced by the appearance of bands in the same positions of the respective zymograms, even suggests that the enzymes are encoded at the same loci in both stages.

Inference of a sporophyte's isoenzyme genotype by analysis of a sample of its gametophytes is based on the following reasoning: If a sporophyte is heterozygous at one isoenzyme locus, then the zymograms expressed by this locus are expected to show 1:1 segregation among the gametophytes. Conversely, observation of 1:1 segregation (*i.e.,* lack of statistically significant deviation therefrom) of two zymograms among a sporophyte's gametophytes is taken as evidence that the sporophyte is heterozygous at exactly one enzyme locus and can make the performance of controlled crosses unnecessary (BARTELS, 1971a and b; BERGMANN, 1973). This quantitative method of inference is, however, sensitive to various forms of segregation distortion (see *e.g.* analysis in GILLET and GREGORIUS, 1992).

Segregation of more than 2 zymograms among a sporophyte's gametophytes indicates the involvement of more than 1 locus. As many as 5 loci have been found to be involved in the expression of enzyme systems, with each heterozygous locus encoding a number of bands depending on the degree of polymerization of the enzyme, *i.e.,* number of enzyme subunits (polypeptides) that make up the completed enzyme (not counting the possibility of interlocus heteromerics or posttranslational modification). For example, for a dimeric enzyme

system encoded at three loci and without interlocus heteromerics, an individual heterozygous at all three loci would possess nine bands (*i.e.,* 2 homomerics and 1 intralocus heteromeric per locus, see "Modes of gene action characteristic of isoenzymes"), and for a tetrameric system 15 (*i.e.,* 2 homomerics and 3 intralocus heteromerics per locus). Quantitative considerations, *i.e.,* calculation of expected haplotype frequencies and comparison with the observed frequencies, for multilocus traits are additionally affected by genetic linkage.

Inference of isoenzyme genotype, which ultimately requires genetic interpretation of each single band in a zymogram, can therefore be difficult. Qualitative analysis consists in the interpretation of each band solely on the basis of its pattern of appearance with respect to the presence or absence of every other band over all of the zymograms. It is sensitive only to the discovery of all different zymograms (*i.e.,* genetic closure of the sample of zymograms as defined in Sec. "Material"). At least for multilocus traits showing discrete variation, such as isoenzymes, qualitative determination of mode of inheritance is the prerequisite for the much less predictable quantitative considerations. (A discussion of the latter is beyond the scope of this paper.)

The objective of this paper is to present the conceptual basis of inheritance analysis of traits in populations in a way that, together with known properties of enzyme structure, will provide computer-implementable rules for the qualitative analysis of the zymograms of tissue expressing only the haploid genetic information of a gametophyte. Although to some the conceptual basis developed in Sec. "The Conceptual Basis of Inheritance Analysis" may seem straightforward, only its consistent application to real traits allows one to overcome the inherent complexity, as is demonstrated in Sec. "Qualitative Inheritance Analysis of Isoenzymes in Haploid Gametophytes". There, implementation of the derived rules provides a procedure, apparently the first to be computer-aided, that systematically generates all hypothetical modes of inheritance conforming to these rules. Certainty is thus provided that alternative interpretations have not been overlooked, the danger of which is exemplified in Sec. "An Example Allowing Numerous Genetic Interpretations". This qualitative method of inheritance analysis is applicable to tissue collected from single individuals as well as from a whole population. It may well be adaptable to other types of traits, such as DNA restriction fragment patterns. Further development may allow interpretation of diplophase zymograms.

An Example Allowing Numerous Genetic Interpretations

An example will demonstrate that the observation of segregation among a single sporophyte's gametophyte zymograms may allow many different genetic interpretations, most of which are liable to be overlooked without the help of a systematic search for hypotheses.

In *figure 1*, the 6 zymograms for the malate dehydrogenase (MDH) system found among 61 haploid primary endosperms of a single tree of the species *Pinus sylvestris* are depicted (MÜLLER-STARCK, personal communication). Since band width and staining intensity do not always reliably reflect amounts of enzyme molecules, and since heteromeric isoenzymes need not be evenly spaced between the corresponding homomerics (cf. RICHARDSON *et al.,* 1986, p. 134), concentration on these quantitative characteristics can be misleading. Accordingly, in *figure 1* spacing between bands and staining intensity is purposely not drawn to scale in order to emphasize the intrinsically qualitative nature of isoenzyme banding patterns. Instead, the gel is partitioned perpendicular to the path of migration into *elementary zones* defined by the positions in the gel where a band appeared in any of the zymograms in the sample. (Usage of the concept "bin" to describe the discrete variation of isoenzyme bands would be misleading, as bins are allowed to contain more than one distinguishable band and are designed for cases of seemingly continuous variation in complex DNA banding patterns (WEIR, 1992). Neither is elementary zone to be confused with the term "zone" used to delimit the range of bands controlled by a single locus.) This practice does not rule out the possibility that two or more enzyme molecules encoded at different loci have identical migration rates (as demonstrated by STÖHR (1992) for 6-PGDH in beech). Consequently, the "same" band appearing in different zymograms, or even a single band in one zymogram, may have more than one genetic interpretation.

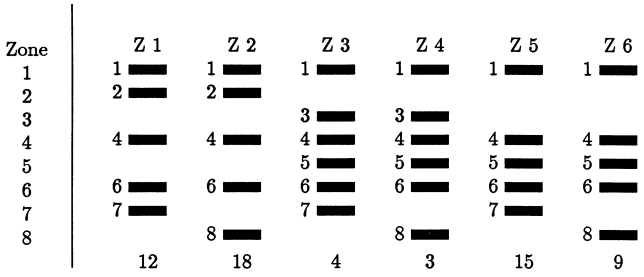

Figure 1. – Schematic diagram (not drawn to scale) of the malate dehydrogenase (MDH) zymograms found among the primary endosperms of tree No. 131 in a stand of *Pinus sylvestris* in Forstamt Grebenau, Hesse, Germany (MÜLLER-STARCK, personal communication). Below each of the zymograms Z1 to Z6, the number of endosperms exhibiting it is noted.

Table 1. – Three hypotheses for the genetic interpretation of the single isoenzyme bands in the 6 zymatograms Z1 to Z6 in *Figure 1*. All 3 postulate a dimeric enzyme system.

Zone	Phenotypes	Type of isoenzyme	Coded by Locus	Allele	Exact test of 1:1 segregation
		Hypothesis 1			
1	Z1–Z6	homomeric	A	A (fixed)	
2	Z1,Z2	homomeric	B	B_1	23:24
5	Z3–Z6	homomeric	B	B_2	$P = 1.00 n.s.$[1]
4	Z1–Z6	homomeric	C	C (fixed)	
6	Z1–Z6	homomeric	D	D (fixed)	
7	Z1,Z3,Z5	homomeric	E	E_1	26:21
8	Z2,Z4,Z6	homomeric	E	E_2	$P = 0.56 n.s.$
3	Z3,Z4	PTM[2] of isoenzyme in zone 5			
		Hypothesis 2			
1	Z1–Z6	homomeric	A	A (fixed)	
2	Z1,Z2	homomeric	B	B_1	23:24
4	Z3–Z6	homomeric	B	B_2	$P = 1.00 n.s.$
6	Z1–Z6	homomeric	C	C (fixed)	
7	Z1,Z3,Z5	homomeric	D	D_1	26:21
8	Z2,Z4,Z6	homomeric	D	D_2	$P = 0.56 n.s.$
4	Z1,Z2	heteromeric	B+C	$B_1 + C$	
5	Z3–Z6	heteromeric	B+C	$B_2 + C$	
3	Z3,Z4	PTM of isoenzyme in zone 4 or 5			
		Hypothesis 3			
1	Z1–Z6	homomeric	A	A (fixed)	
4	Z1,Z2	homomeric	B	B_1	23:24
5	Z3–Z6	homomeric	B	B_2	$P = 1.00 n.s.$
6	Z1–Z6	homomeric	C	C (fixed)	
7	Z1,Z3,Z6	homomeric	D	D_1	26:21
8	Z2,Z4,Z6	homomeric	D	D_2	$P = 0.56 n.s.$
2	Z1,Z2	heteromeric	A+B	$A + B_1$	
4	Z3–Z6	heteromeric	A+B	$A + B_2$	
3	Z3,Z4	PTM of isoenzyme in zone 4 or 5			

[1]) *n.s.* = not significant, $P \geq 0.05$;
[2]) PTM = post-translational modification

The following qualitative observations characterize the 6 zymograms in *figure 1:* Three zones (1, 4, 6) are *fixed*, *i.e.,* contain a band in every zymogram. A band always appears in either zone 2 or 5 but never in both. The same holds true of zones 7 and 8. A band only appears in zone 3 when zone 5 is also occupied. *Table 1* lists 3 possible hypotheses for the genetic interpretation of the zymograms in *figure 1.* Note that in hypotheses 2 and 3, the seemingly fixed zone 4 has 2 different explanations. The observed frequencies of the zymograms show no statistically significant deviation from any of the 3 hypotheses.

The computer program HAPLOZYM described at the close of this paper additionally formulated many more hypotheses, all of which postulate identical migration rates for molecules with differing subunit structures and most of which either require null alleles at one or more loci or predict the existence of additional banding patterns that were not found among the 61 endosperms. The probability of having missed gametophytes possessing these additional patterns during sampling depends on the frequency of the rarest pattern and the sample size (see GREGORIUS, 1980, for calculation of requisite sample size).

The example shows that segregating endosperm zymograms may be interpretable both qualitatively and quantitatively by more than one hypothesis, with alternative hypotheses being easily overlooked. Analysis of endosperms of other trees from the same population supported hypothesis 2 (MÜLLER-STARCK, personal communication). In fact, the MDH system in *Pinus sylvestris* appears to be one of the most difficult for inheritance analysis in conifers (cf. EL-KASSABY, 1981), as is apparent from the persistence of contradictory interpretations (RUDIN and EKBERG, 1978; and others; MÜLLER-STARCK, 1984, 1987; and personal communication; MEJNARTOWICZ and BERGMANN, 1985). Nevertheless, new problems continually being presented by the growing number of species and enzyme systems studied urge the formulation of a comprehensive conceptual basis for analysis.

The Conceptual Basis of Inheritance Analysis

Denote any observable property of conspecific individuals (*e.g.,* flower color, height, isoenzyme banding pattern) as a *character.* The purpose of *inheritance analysis* of characters possessed by a given set of conspecific individuals is to determine the *mode of inheritance* as the relationship between each individual's genetic information (genotype) and its characters (phenotype). One of the fundamental tenets of genetics is that the expression of every character in an individual is governed by the genetic information of the individual and the individual's environment; no other factors exist. The actual task of inheritance analysis (cf. GREGORIUS, 1977) is thus to determine the norm of reaction of each genotype, *i.e.,* "the range of potential phenotypes that a single genotype could develop if exposed to a specified range of environmental conditions" (WOLTERECK, 1909; cit. RIEGER *et al.,* 1991). This task is seemingly complicated by the consideration that the expression of all characters is ultimately dependent on the environmental conditions in which the individual is situated; in extreme environments an individual may not be able to survive long enough to express any character at all (GIFFORD, 1990). Thus all statements about the role of genetic information in character expression must be restricted to the set of environments included in the investigation.

The pathway for inference of the mode of inheritance, which comprises the mode of transmission and mode of gene action (see below), is provided by observation of the norms of reaction possessed by individuals and their offspring and the attempt to explain the inheritance of reaction norms as the effect of a redistribution of genetic units by the processes of meiosis and gametic fusion. Two prerequisites for the utilization of this pathway are

P1: the unambiguous definition of the characters under study and the environments in which they are observed, and

P2: translation of the principles of meiosis into a form that reflects their operational implications for transmission of genetic information to gametes.

In the following sections, the general principles of inheritance analysis as presented *e.g.* in BERGMANN *et al.* (1989) are complemented by the introduction of new concepts called for in the previous paragraph.

Traits

In fulfillment of P1, a set of characters of the members of a collection of individuals defines a *trait* of the collection over a given set of environments, if each member expresses one, and only one, of these characters in each environment. In this case, the character expressed by each member unambiguously defines its *trait state* in the respective environment. The different trait states together specify the *trait variable.*

A set of characters will often define a trait only after the characters and the environmental conditions of the individuals are more precisely described. To ensure uniqueness, character description may specify nuclear phase (haplo-, diplophase), ontogenetic stage, tissue type, etc. Environmental conditions include the biotic and abiotic environment of the live individual as well as, in an extended sense, laboratory protocols for study of its tissues, if applicable; for traits expressed as electropherograms (*e.g.,* DNA and isoenzyme banding patterns), differences in tissue extraction and storage, variation of buffer or gel characteristics, staining techniques, etc., may change the banding patterns.

The definition of a trait thus requires specification of a collection of individuals, description of a set of characters of the members of the collection including the environmental conditions under which the characters are observed, and verification that each member of the collection exhibits exactly one character in each environment.

In the strict sense, a trait of a given collection of individuals is termed a *genetic trait* in a given set of environments, if the trait state that each organism expresses in its original environment does not change when the organism (or a genetically identical copy) is placed into any of the other environments; the norm of reaction for each genotype is thus a constant mapping over the set of environments. Alternatively, trait state invariance over the environments can be replaced by the less stringent requirement that the effects of the genetic information on the organisms' trait states be separable from the environmental effects, thus guaranteeing consistency of the genetic effect (GREGORIUS, 1977; GREGORIUS and NAMKOONG, 1986, 1987). Note that the application of both definitions ideally requires placement of genetically identical copies of each of the members of the collection of organisms into each of the environments.

Units of inheritance

The unit of inheritance is usually termed "gene". The existing imprecision in current usage of this term is a well-known problem, however. It is easy to find new publications in which "gene" alternately signifies locus (as in "multigene family") on the one hand and a particular DNA sequence with a defined function (as in "mutant gene") on the other. This confusion may trace back to the similarly ambiguous usage of the

terminological predecessor "genetic factor" *e.g.* in MORGAN *et al.* (1922, especially in Chapter IX). But what does one call a single segment of a single individual's chromosomal DNA, *i.e.,* the actual molecular carrier of a piece of genetic information, irrespective of its specific DNA sequence? An unambiguous term is needed, since the considerations presented below begin at the elementary level of single individuals and gametes rather than classes of genetically identical ones. After much deliberation and because identical transmittability of the genetic information rather than its function is of main interest, the following term was chosen:

Define a *transmitton* in an individual to be a single segment of DNA in the individual (and not the set of all segments with identical DNA sequence) that with high probability is transmitted via DNA replication either in its entirety to any of the individual's gametes or not at all. To avoid ambiguity in multicelled organisms, the transmittons of an individual will be defined in the zygote from which it developed. By tracing the identical copies of each transmitton by mitotic division through an individual's cell lines, one can also speak of the transmittons of a cell such as a meiocyte. As a result of this convention, all of an individual's meiocytes can be thought to contain the "same" transmittons. By DNA replication, a transmitton in a gamete is a *copy* of a parental transmitton.

A transmitton represents a unit of inheritance. As it need not have a physiological function in the traditional sense, it will in general not coincide with a unit of function. If it comprises more than 1 unit of function, these units of function are said to be completely linked; the opposite case, where a unit of function has an internal recombination site and thus consists of more than 1 transmitton, allows for so-called intragenic recombination. Indeed, many (if not most) transmittons encompass or even entirely consist of non-coding DNA. Yet perhaps the apparent discrepancy between the unit of inheritance and the unit of function can be resolved by considering that even non-coding DNA has the "function" of becoming visible as a band in an electropherogram or as a series of bands in a sequencing gel when very specific laboratory techniques (*i.e.,* environmental conditions) are applied.

Mode of transmission

The *mode of transmission* as 1 component of the mode of inheritance "specifies the mode according to which … genetic units [our transmittons] are transmitted by an individual to its offspring" (BERGMANN *et al.,* 1989). Any attempt to specify mode of transmission (and be it only for the sake of proving that a transmitton is not nuclearly transmitted) must be based on the most important concept in inheritance analysis, that of "transmission homology". In this section, the terms "locus", "degree of ploidy", and "linkage" usually used to describe modes of transmission will be seen to follow effortlessly from a careful development of this concept. (The adjective "transmission" distinguishes this type of homology from the many other unrelated uses of this term.)

Using modern terminology, we are now certain that transmittons are organized in linear order in DNA molecules which, when located in nuclei, appear in chromosomes having homologous counterparts in individuals of ploidy ≥ 2, and that homologous (usually recombined) chromosomes are separated during meiosis. This structuring among nuclear transmittons, as we know it today, places severe restrictions on the transmission of homologous transmittons to the same meiospore. MENDEL (1866) was probably the first to conclude that the transmission of one transmitton (his *Zellelemente* on p. 42) through a gamete (his *Befruchtungszelle*) to an offspring always precludes the transmission of a certain other transmitton through the same gamete. Thus he fulfilled P2 by discovering one of the major consequences of the mechanism of meiosis, the concept of transmission homology formulated in the following paragraphs.

In effect, MENDEL inferred the existence of sets of transmittons in each individual that have the following properties, formulated here for organisms of degree of ploidy ≥ 2:

Completeness, meaning that each of the individual's gametes contains a copy of at least one of these transmittons.

Complementarity, in that copies that are included in any one of the individual's gametes are complemented in a second of its gametes, in the sense that the two gametes do not share copies of the same transmitton and the union of their copies equals the entire set.

Minimality, in that it is complete and possesses complementarity, and no proper subset has these properties.

A minimal set of transmittons is termed a *set of homologous transmittons in the individual.* Two transmittons from the same set will be called *transmission homologous.*

The following terms owe their existence to the basic concept of transmission homology. Each set of transmission homologous transmittons describes a *locus,* and 2 transmission homologous transmittons are *allelic.* The number of transmittons in a given set of transmission homologous transmittons in an individual defines the *degree of ploidy* of the individual with respect to this set.

If a set of transmission homologous transmittons consists of only 2 transmittons, *i.e.,* if the individual is *diploid* for this set, then the above properties reduce to the following:

Two transmittons in a diploid individual form a set of transmission homologous transmittons, if the individual transmits either one (completeness) but never both (mutual exclusion, ensuring complementarity and minimality) to each of its gametes, that is, if each of its gametes possesses exactly one of them.

A set of transmittons sometimes cannot be made complete by adding other transmittons due to deletion of the respective DNA segment in one of the individual's ancestors. Such a "deleted transmitton" can be construed to be transmission homologous to a second transmitton, if a set of transmittons containing the two of them fulfills the properties of a set of transmission homologous transmittons. The familiar example of the "null allele" in isoenzyme analysis is referred to later.

Up to now, only transmittons of a single individual have been considered where each transmitton is a unique entity different from any other transmitton. In order to extend the concept of transmission homology to a whole collection of individuals, transmittons of different individuals must be made comparable by considering some trait definable for the set of all transmittons (*i.e.,* each transmitton has exactly one trait state). Frequently considered traits of transmittons are identity by descent, identity of DNA sequence, and identity of function. Two transmittons a and b are then said to be of the same *type* (denoted $a \sim b$), if they possess the same trait state. Note that a trait defines an equivalence relation on the set of transmittons, since for all transmittons a, b, c the following properties hold: $a \sim a$ (reflexivity), $a \sim b$ if and only if $b \sim a$ (symmetry), and if $a \sim b$ and $b \sim c$, then $a \sim c$ (transitivity). If 2 transmittons at the same locus in an individual are of the same type, then they both represent the same *allele* at the locus.

Relationships between transmittons, types and loci can be rather complex. Two transmittons at a locus in an individual may be of the same type or of different types. In the first case,

the individual is *homozygous* at the locus, in the second *heterozygous.* (At the extreme, if the trait state of each transmitton is its unique identity, then no 2 transmittons of the same individual can be of the same type.) Transmittons of the same type in an individual may also belong to different loci, as for transmittons at duplicated loci. (In fact, 2 loci can be defined to be related by duplication, if they share transmittons of the same type; as it should be, the statement of duplication is thus dependent on the trait under consideration.)

Now suppose that a locus has been identified in each individual member of a collection. What does it mean to say that the different loci in the members all define the "same" locus? As long as we cannot somehow bring the genetic information of two members together and study fulfillment of the properties of transmission homology in this new member, we cannot hope to judge whether the loci are the same. After all, higher organisms contain sets of functionally similar (with respect to function duplicated) loci. The solution to this problem can best be introduced by the following considerations.

Such a collection-wide locus surely must be identified with the (set theoretic) union of all transmittons belonging to the corresponding sets of transmission homologous transmittons in the members. It is reasonable to require that, if transmittons of any two members "come together" in a third member of the collection, then they must be transmission homologous in this member also. The problem here is that one transmitton as a unique entity cannot be present in two members. For this reason, it is necessary to find a third member containing transmittons of the same types rather than the transmittons themselves. But how many transmittons can actually appear in any third member? Certainly only as many as can be transmitted by two gametes, since every member is the product of gametic fusion. Finally, what if the collection is too small for every possible "third" member to actually exist? Then we cannot be sure whether the loci are indeed the same. Specification of a third member may in fact require enlargement of the collection by including offspring of some of the original members, thus relating the present concept to the methods of classical MENDELian analysis.

The above considerations lead to the following definition, which due to its complexity can best be presented as a procedure: The union of one set of transmission homologous transmittons per member of a collection defines a set of *transmission homologous transmittons (locus) in the collection,* if the following steps are fulfillable for any pair of gametes producible by any pair of members:

T1: for each gamete, determine which of the transmittons of its producer at the respective locus were transmitted to it,

T2: identify the type of each of the transmittons of T1, and

T3: find a third member of the collection, such that the set of types represented by this third member's set of transmittons at its locus exactly equals the set of types constructed in T2.

(Any 2 or all 3 members may be the same individual.) The concept of allele can now be unambiguously extended from the individual to the collection to signify a type of transmitton at a given locus.

Application of the concept of genetic trait to transmission homology in a collection yields more familiar terminology. For a given locus in a collection of individuals, the alleles at the locus specify a genetic trait as follows: The set of alleles possessed by an individual defines a character of the individual, termed its *genotype* at the locus (or *haplotype*, if the individual is haploid). Since each individual has a single genotype at this locus (specification of a particular tissue type may be necessary), the genotypes of the members of a collection form a trait in the above-defined sense. Neglecting the possibility of mutation, these genotypes remain invariant over all environments in which the individuals can survive and thus describe a genetic trait in the strict sense. For a defined set of loci, the multilocus genotypes of the conspecific individuals in a collection also define a genetic trait in the collection.

Expressed in this terminology, the condition guaranteeing that loci in individuals form a locus in the collection simply means that each pair of haplotypes among the gametes of the collection appears as the genotype of some (diplophase) member of the collection.

Mode of gene action

The *mode of gene action* as the other component of the mode of inheritance of genetic trait variation specifies "the interaction of the functional genetic units or subunits involved in the generation of the trait expression" (BERGMANN *et al.,* 1989), our trait states. Here, not the transmitton itself but rather the function of the transmitton, as expressed by its type (see above), is of interest. Functions can be complex, such as the encoding of a particular molecular variant (*e.g.,* isoenzyme subunit), or as simple as its containment of a restriction fragment of a particular length. Notwithstanding the danger of misinterpretation mentioned above, the term *gene* will now designate a transmitton together with its type. Clarification of the mode of gene action allows each genotype to be assigned the trait state that it produces or, equivalently, each trait state to be associated with a set of one or more genotypes.

A genetic trait for which every trait state is produced by only one genotype is termed a *gene marker,* because the genes are identifiable. This requires a codominant mode of gene action as, for example, in isoenzymes (in the absence of null alleles). A genetic trait possessing a dominant mode of gene action (as do most RAPD markers) is not a gene marker. Gene markers play a special role in the field of population genetics, for example, as they allow direct estimation and comparison of allelic and genotypic frequency distributions in populations.

Beyond the familiar concepts of dominance/recessivity, codominance, and epistasis (which also comprises specification of the mode of transmission by indicating the transmission homology or non-homology of the transmittons), a general description of modes of gene action does not allow the degree of abstraction that we have seen to be possible in the discussion of the mode of transmission, as the types of interaction that can be observed between genes depend heavily on the trait in question (GREGORIUS and NAMKOONG, 1987). Modes of gene action characteristic of isoenzymes will be discussed later.

Inheritance analysis of trait variation

As introduced above, the inheritance analysis of variable genetic traits consists in the formulation and application of a method enabling determination of the mode of inheritance of genetic trait variation. It can be divided into three steps:

I1: finding a method that enables identification of each trait state with a set of genes,

I2: affirmation that the constituent genes actually possess the characteristics of transmittons as units of inheritance, and

I3: verification that the set of genes fulfills the requirements of transmission homology by describing one or more loci.

Proof of gametophytic origin of genetic trait variation in sporophytes

Inheritance analysis using the primary endosperm of conifer seeds is based on the cytologically founded observation that the

genetic information possessed by the primary endosperm is identical to the information that is transmitted to the zygote (seed embryo) through the maternal gamete. This observation can be confirmed by examining the offspring of controlled crosses of a single tree as the maternal parent with a number of pollen donors (fathers) possessing differing trait states. If only the maternal contribution is being observed, then the frequency distributions of the different trait states within each full-sib progeny set should not show statistically significant differences between sets (hypothesis of homogeneity), except perhaps under some form of segregation distortion (see below). Ideally, this test must be repeated for each mother using the same set of fathers, as dominance of the maternal genes could conceivably have masked the activity of paternal genes in the initial crosses.

Can any of the various forms of segregation distortion (cf. GILLET and GREGORIUS, 1992) cause significant differences in the distribution of maternally transmitted trait states between progenies of the same mother and different fathers? Gametic viability selection (including meiotic drive) among maternal gametes will not, since the distortion arises prior to fertilization and is thus repeated in the progenies of all crosses. Neither will gametophytic incompatibility as a heterophasic type of mating system (GREGORIUS, 1989, p. 130) cause differences, since the distortion affects only the male gametic contribution. Differences may, however, result under a haplo-homophasic system of mating (selective fertilization), in which the fusion of a pair of gametes is solely determined by haplophase characters of these gametes (GREGORIUS, 1989, p. 130), and in the case of differential post-zygotic viability selection prior to observation.

Even when limited to a small number of individuals, such testing can be time-consuming. Therefore, it has become common practice to incorporate the hypothesis of maternal transmission into formulation of the mode of transmission of a genetic trait and then test the resulting hypothesis. In conifers, the usual test of 1:1 segregation of the putative alleles at each postulated locus among the seed endosperms from open pollination of single, presumably heterozygous mothers simultaneously tests the hypothesis of maternal transmission. Rejection of correct hypotheses may nevertheless result *e.g.* due to haplo-homophasic mating and differential post-zygotic viability selection, as explained above. In fact, reports of segregation distortion in doubled haploid crop plants (THOMPSON *et al.,* 1991) and poplar (STOEHR and ZSUFFA, 1990) produced by microspore culture question the validity of rejecting a postulated mode of inheritance on the basis of distorted segregation ratios.

Qualitative Inheritance Analysis of Isoenzymes in Haploid Gametophytes

As an application of the conceptual basis of inheritance analysis described above, qualitative rules for the genetic interpretation of the single isoenzyme bands in the zymograms of haploid gametophytes will be derived. A computer program (HAPLOZYM) based on these rules is introduced that systematically searches for hypotheses on the mode of inheritance of the zymograms.

This method also facilitates determination of the mode of inheritance of isoenzymes expressed in diploid tissue. Prerequisites are that the genes encoding the enzyme subunits in the gametophyte are also active in the diploid tissue and that the zymograms describe a genetic trait in the collection of diplophase individuals and in the collection of gametophytes.

Modes of gene action characteristic of isoenzymes

In brief review, enzyme molecules consist of one or more subunits (polypeptides, the number depending on the degree of polymerization, see below), each encoded by a gene (one gene – one polypeptide hypothesis). Two transmission homologous genes (as transmittons) represent the same *allele* at the locus, if the subunits they encode have identical effects on the migration velocity of the completed enzyme through a gel (*i.e.,* functional equivalence of the genes), and they represent different alleles if their effects are discernible. Because each genotype at a locus thus corresponds to a unique phenotype identifiable as one or more isoenzyme bands, the usual mode of gene action of isoenzymes is codominance.

An exception to codominance can occur if a *null allele* exists at a locus, *i. e.,* a gene that fails to encode functional enzyme subunits under the environmental conditions of the experiment. Causes of failure can range from a defect in the DNA sequence prohibiting its transcription or leading to non-detectability of its product to (epistatic) inhibition of gene expression by regulatory molecules or even to prior deletion of the coding DNA sequence. A null allele is present if no homomeric enzyme molecule is detectable under the given experimental conditions. Not every null allele need be recessive to "active" alleles (*i.e.,* alleles producing a homomeric molecule), however, because heteromerics combining products of a null allele and an active allele may still be functional, in which case codominance is retained. (JABLONKA's and LAMB's (1989) criticism of the term "active" allele is not relevant here, since it is applied to a gene that is functional in the context of the experiment.)

The degree of polymerization is an additional component of the mode of gene action of isoenzymes. In polymeric enzyme systems as opposed to monomeric systems, enzyme molecules consist of at least two subunits (two for a dimeric system, four for a tetrameric, etc.). Enzyme molecules are termed *homomeric* if all of their subunits are identical, *i.e.,* are encoded by functionally equivalent genes. (It is possible that 2 genes at different loci are functionally equivalent, in which case an enzyme could be an interlocus homomeric, but such a molecule would have the same migration velocity as and thus be indistinguishable from the corresponding intralocus homomeric.) *Intralocus* and *interlocus heteromeric* enzyme molecules consist of non-identical subunits encoded by different alleles of one locus (implying that the individual is heterozygous at the locus) or functionally nonequivalent genes at different loci, respectively. For convenience, the isoenzymes of monomeric systems will be subsumed under the homomerics, as they necessarily consist of only one subunit type.

Post-translational modification (PTM) can result in alteration of the electrostatic charge or molecular conformation of an enzyme molecule in a way that affects its migration velocity through the gel. PTM is presumably caused by epistatic effects of genes at a second locus under certain environmental conditions and, if not all molecules of a particular subunit structure in an individual are modified, results in the appearance of one or more additional bands in the zymogram. Even though genes causing PTM actually participate in the genetic control of the zymograms, they are usually relegated to the "genetic background", since they do not themselves encode subunits of the enzyme system under study. Two types of PTM of molecules of a given subunit structure can be distinguished within a collection of individuals in its environment: A PTM of a particular molecule will be termed *fixed,* if the PTM occurs in all members possessing the molecule. Otherwise, if variation at these "background" loci causes PTM of the molecule in some of

the members of the collection that possess it but not others, the PTM will be termed *facultative.*

Physico-chemical alteration of enzymes in extreme environments, a rare natural occurrence, separates these environments from "normal" environments within which zymograms define a genetic trait and thus will not be considered here.

Material

Assume in the following that the zymograms of the enzyme system under study constitute a genetic trait in a collection of gametophytes. The environmental conditions, including laboratory procedures, are assumed to be the same for all gametophytes; only this can guarantee the comparability of the zymograms. Additionally assume that the given sample of gametophytes is *genetically closed,* in that it contains all possible genotypes (here haplotypes) that can arise by association of the alleles at all loci encoding subunits of this enzyme system in the sample.

Genetic closure is of course not recognizable at the outset of an investigation, but sampling strategies can be devised that take advantage of known genealogical relationships among the individuals in the collection. For example, a large sample of gametophytes from a single sporophyte will have good chances of being genetically closed. The same holds for a large, bulked sample of gametophytes from a population, if it can be assumed that the population is genetically closed with respect to the (diplophase) genotypes of the sporophytes.

An additional assumption, which can be circumvented (see description of program), is that enzyme molecules encoded by different genes or, in the case of heteromerics, sets of genes always have discernibly different migration rates.

The method

Given such a set of zymograms, qualitative inheritance analysis satisfying steps I1 to I3 above consists in interpretation of the patterns of appearance of bands in the elementary zones. These patterns are classified as follows. An elementary zone is *fixed,* if a band appears in this zone in all of the zymograms. One non-fixed elementary zone is termed *dependent* on a second non-fixed elementary zone, if a band appears in the 1 zone of any zymogram only if a band is also present in the second. A non-fixed elementary zone *i* is *independent,* if it is not dependent on any other elementary zone, *i.e.,* for each other non-fixed elementary zone *j,* there exists a zymogram with a band in *i* but no band in *j.* Two elementary zones are in *complete correspondence,* cases of which are listed in *table 2,* if each is dependent on the other or if both are fixed. To avoid unnecessary complication, completely corresponding elementary zones are grouped together in the following into one extended elementary zone, the "fixed zone". It will be argued that the genetic interpretation of each elementary zone can be inferred from its manner of dependence on other elementary zones. The fixed zone forms the only exception, since its lack of variation prohibits its interpretation.

The 1 gene – 1 polypeptide hypothesis implies that the subunits of a homomeric isoenzyme are all encoded by genes of the same type at a single locus. (For convenience, monomeric isoenzymes are referred to as homomerics.) For the time being, the probably rare case of duplication as defined above is ruled out. The strategy of the method is to satisfy I1 by identifying the elementary zones containing homomeric isoenzymes as follows: A homomeric encoded by a gene present in all zymograms appears as an uninterpretable fixed band. Non-fixed elementary zones containing homomerics are independent due to the genetic closure of the set of zymograms. It remains to show that all independent zones contain homomerics. To do this, it will be demonstrated that non-fixed elementary zones that do not contain a homomeric are dependent (with 1 rare exception).

Interlocus heteromeric isoenzymes are encoded by 2 (or more?) genes, each of which also produces a homomeric isoenzyme. (Intralocus heteromeric isoenzymes cannot be formed in haploid tissue.) This corresponds to codominance as the mode of gene action. Consequently, the appearance in a zymogram of bands representing heteromerics depends on the appearance of bands representing the corresponding homomerics. Furthermore, appearance of a band in an elementary zone representing a PTM is dependent on the appearance of the unmodified isoenzyme (as long as not all molecules are modified and the zone of the unmodified isoenzyme is not fixed). This dependence distinguishes the elementary zones of heteromerics and PTM's from those of homomerics. The single, probably rare case of an independent zone that is not homomeric is given by a facultative PTM of a fixed zone.

Thus having identified genes by distinguishing independent zones, I1 above is fulfilled. Later confirmation of lack of "intragenic recombination" among the gametophytes of single individuals may be necessary to affirm I2.

Now considering only the independent elementary zones, the assignment of the underlying genes to loci in fulfillment of I3 follows according to the concept of transmission homology. In particular, the homomeric elementary zones corresponding to a locus are recognizable by the appearance of a band in exactly one of them in each zymogram (transmission homology for diploidy). If a set of homomeric zones cannot be made complete, then the existence of a (recessive) null allele at the locus must be postulated. However, care must be taken if a locus is found to comprise only one active allele and a null allele; an alternative explanation is that the "active allele" is a facultative PTM of an isoenzyme in the fixed zone, which, as mentioned above is the only case of an non-homomeric independent zone. For interpretation of dependent elementary zones, see *table 3.*

It is interesting to note that one of the results of application of the conceptual basis of inheritance analysis (Sec. "The Conceptual Basis of Inheritance Analysis") to a genetically closed sample of gametophytes is that knowledge of enzyme subunit structure as given by the degree of polymerization (as a component of the mode of gene action) does not facilitate qualitative inference of the mode of transmission of isoenzymes. The mode of transmission is completely specified once the homomeric isoenzyme bands have been identified and assigned to the alleles of various loci. Homomerics, in turn, are recognizable by their independence of the appearance of any other bands, with mutually exclusive bands corresponding to alleles of the same locus.

Table 2. – Cases of complete correspondence between elementary zones. In these cases, the elementary zones are combined into 1 "extended" elementary zone.

- ▹ One zone contains a homomeric isoenzyme and the other a fixed PTM of it.
- ▹ One zone holds a homomeric isoenzyme and the other zones heteromeric isoenzymes formed from the subunits encoded by the gene of the homomeric and the gene at another locus that is fixed in the collection of gametophytes.
- ▹ In a polymeric enzyme system of degree > 2, zones contain interlocus heteromeric isoenzymes encoded by the same two genes but differing in the number of polypeptides encoded by each.
- ▹ The zones are fixed, in that they contain homomerics of fixed loci, interlocus heteromerics between fixed loci, or fixed PTM's of either.

Table 3. – Properties of the elementary zones representing the various types of isoenzyme under a codominant mode of gene action including the possibility of null alleles. Migration velocities of molecules encoded by different genes or sets of genes are assumed to be unequal. These properties hold in a genetically closed sample of gametophytes. (See text for explanation of terminology).

	Type of isoenzyme	Property of elementary zone
	Unmodified enzymes and fixed PTM's[1]	
I.	Homomeric of gene at variable locus together with its fixed PTM's	Independent zone
II.	Homomeric of gene at fixed locus together with its fixed PTM's	Fixed zone
III.	Heteromeric between genes at variable loci together with its fixed PTM's	Zone dependent only on the two independent zones of the resp. homomerics
IV.	Heteromeric between gene at a variable locus and gene at a fixed locus together with its fixed PTM's	Is represented by the (extended) homomeric zone of the gene at the variable locus
V.	Heteromeric between genes at two fixed loci together with its fixed PTM	Fixed zone
	Facultative PTM appears as additional band	
	Homomeric (I) and PTM	Zone of PTM dependent on independent zone of unmodified homomeric
	Homomeric (II) and PTM	Unmodified homomeric is fixed band, PTM is independent zone that defines trait with no other independent zone
	Heteromeric (III) and PTM	Zone of PTM dependent on dependent zone of unmodified heteromeric
	Heteromeric (IV) and PTM	Zone of PTM dependent on zone of homomeric of variable locus
	Heteromeric (V) and PTM	Unmodified heteromeric is fixed band, PTM is independent zone that defines trait with no other independent zone

[1]) PTM = post-translational modification

The computer program HAPLOZYM

The method outlined in the previous section is implemented as a computer program. The input to the program is a schematic representation of the different zymograms observed in a genetically closed sample of gametophytes. The zymograms are represented as follows: As in *figure 1,* the different elementary zones are numbered consecutively. If there are N elementary zones, then each zymogram can be expressed as a list of N 0's and 1's, with a 1 in the mth position signifying that the isoenzyme in the mth zone appears in this zymogram and a 0 that it does not. For example, zymogram Z1 in *figure 1* can be written as 11010110. An option allowing specification of subunit structure is not provided, since it would needlessly restrict the set of alternative hypotheses without simplifying calculations (see previous paragraph).

Hypotheses are derived as described above. The number of zymograms that should exist under each hypothesis is compared with the number found as a control of the genetic closure of the sample. Because the assignment of independent zones to gene loci can depend on the ordering of the elementary zones in the input data, all permutations of the input data can optionally be tested for the existence of additional hypotheses.

If no hypothesis can be formulated, one reason may be violation of the above assumption that enzyme molecules encoded by different genes or sets of genes have different migration rates. HAPLOZYM optionally provides for this possibility by consecutively substituting each elementary zone by 2 new zones such that each zymogram that exhibits a band in the original zone is redefined in 3 ways: as possessing a band in (1) the one new zone but not the other, (2) the second but not the first, and (3) both new zones. Each of the 3^k combinations arising from the 3-fold redefinition of each of the k zymograms possessing a band in the original zone is generated, and the original set of zymograms is redefined accordingly. HAPLOZYM treats each redefined set of zymograms as new input. In the program's present form, only 1 zone can be "split" at a time.

The Fortran 77 source code of HAPLOZYM is available from the author (E-Mail: gillet@ufogen.uni-forst.gwdg.de).

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Giemsa C-Banding Pattern of the Chromosomes in the Macrogametophyte of Norway Spruce

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Abstract

Giemsa C-banding method was applied on chromosomes in the female gametophyte tissue of Norway spruce (*Picea abies* (L.) KARST.). A karyogram of 1 single tree growing in the Botanical Garden of the University of Graz, Austria, was made by using an image analysis system. All chromosomes (n = 12) showed pericentric C-bands. Chromosomes II, V and IX possessed intercalary C-bands located at secondary constrictions. Telomeric C-bands appeared in chromosomes II and III on both arms. Stickiness between non-homologous chromosomes was observed and discussed in the paper.

Key words: Picea abies (L.) KARST., chromosomes, Giemsa C-banding method, macrogametophyte, stickiness, karyotype, image analysis.

FDC: 165.3; 174.7 *Picea abies.*

Zusammenfassung

Die Giemsa-C-Bänderungsmethode wurde an Makrogametophyten der Fichte (*Picea abies* (L.) KARST.) durchgeführt. Das Karyogram eines Baumes aus dem Botanischen Garten der Universität Graz, Österreich, wurde unter Zuhilfenahme eines Bildanalysesystems erstellt. Alle Chromosomen (n = 12) zeigten perizentrische C-Banden. Die Chromosomen II, V und IX weisen interkalare C-Banden, die an den sekundären Einschnürungen liegen, auf. Telomerische C-Banden wurden an den Chromosomen II und III an beiden Chromosomenarmen gefunden. Sticky-Effekte zwischen nicht homologen Chromosomen wurden beobachtet und ihr Auftreten wird in dieser Arbeit diskutiert.

Introduction

Karyotyping of gymnosperm macrogametophytes (= female gametophyte tissue, endosperm tissue in development) has been rarely used, but it does offer advantages in the preparation and finding of well-spread metaphase chromosomes. This haploid endosperm tissue in development was investigated by SAX and SAX (1933), SANTAMOUR (1960), PEDERICK (1967, 1970), BORZAN (1977a and b, 1981, 1988), BORZAN and PAPEŠ (1978), MACPHERSON and FILION (1981) and by some other cytogeneticists. Norway spruce (*Picea abies* (L.) KARST.) was investigated by SAX and SAX (1933), SANTAMOUR (1960) and KÖHLER et al. (1995).

Giemsa C-banding method was applied to gymnosperm chromosomes by BORZAN and PAPEŠ (1978), MURATOVA (1978), BORZAN (1981, 1988), MACPHERSON and FILION (1981), TANAKA and HIZUME (1980), WOCHOK et al. (1980). TEOH and REES (1977) applied this method to root tips meristem chromosomes of white spruce (*Picea glauca*) illustrating one A chromosome with pericentric C-band and B chromosome with no Giemsa banding.

In order to improve the identification of Norway spruce chromosomes, we have opted for the application of Giemsa C-banding method to the endosperm tissue in development of 1 Norway spruce tree of unknown origin, growing at the Botanical Garden of the University in Graz. The processing of data was possible by digital image analysis, and the revealed karyotype can be used as a reference for comparison with Norway spruce karyotype analyses from studies to detect intraspecific variation and interspecific differences.

Materials and Methods

Plant material, ovules fixation and slide preparation

During May, cones from a single tree of Norway spruce were collected. Ovules were extracted, fixed in 3:1 ethanol : acetic acid for 24 hours at room temperature and stored in 70% ethanol at 4 °C.

Stored macrogametophytes were sequentially placed in 50% and 30% ethanol for 10 minutes. After rinsing in distilled water they were placed in 45% acetic acid for 15 minutes. A small incision was made through the integument, the endosperm teased and gently squashed in 45% acetic acid. The coverslip was removed using liquid nitrogen. The slides were dehydrated for a few minutes in 96% ethanol and dried at room temperature overnight.

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