Using molecular marker technology in studies on plant genetic diversity

Protein-based technologies

Isozymes

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  - Lima bean
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Detecting isozymes: methodology

- Pre-treating plant material
- Starch or acrylamide gel electrophoresis
- Histochemical staining
- Analysing banding patterns

Crude tissue extracts are subjected to gel electrophoresis and probed with enzyme-specific stains, resulting in a simple banding pattern.

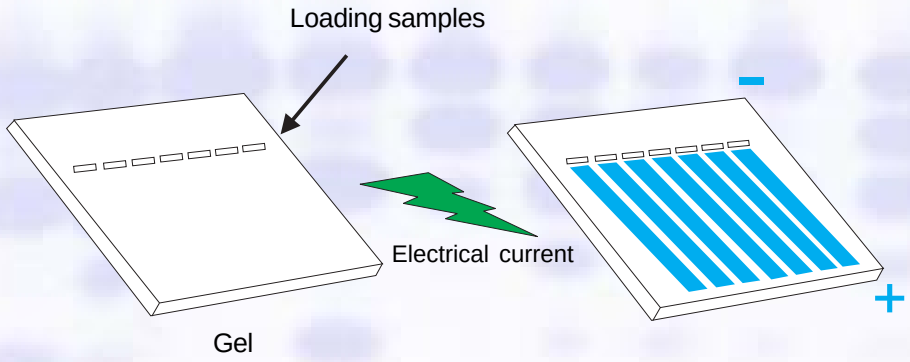
Variation in banding patterns between individuals can be interpreted genetically, as would be done with any other phenotypic marker.

Detecting isozymes: gel electrophoresis (1)

- This process combines the separation of molecules by charge with that by size by applying an electric current
- The current makes the molecules move through pores in a layer of gel
- The substance that makes up the gel is selected so its pores are of the right size to separate a specific range of molecule sizes and shapes

Electrophoresis is a chromatographic technique for separating mixtures of ionic compounds. It has been adapted as a common tool for biochemical analysis.

Detecting isozymes: gel electrophoresis (2)



Proteins are the primary product of genes. When the nucleotide sequence of the DNA changes, so too do the proteins' banding patterns. Enzyme electrophoresis can directly reveal genetic polymorphism through demonstrating the multiple forms of a specific enzyme.

Equipment

- Resources:
  - Distilled and/or deionised water
  - Reagents

- Equipment:
  - Refrigerator and freezer
  - Power supply units
  - Hotplate or microwave
  - Thick cotton gloves
  - pH meter
  - Balances
  - Gel units
  - Suction and volumetric flasks

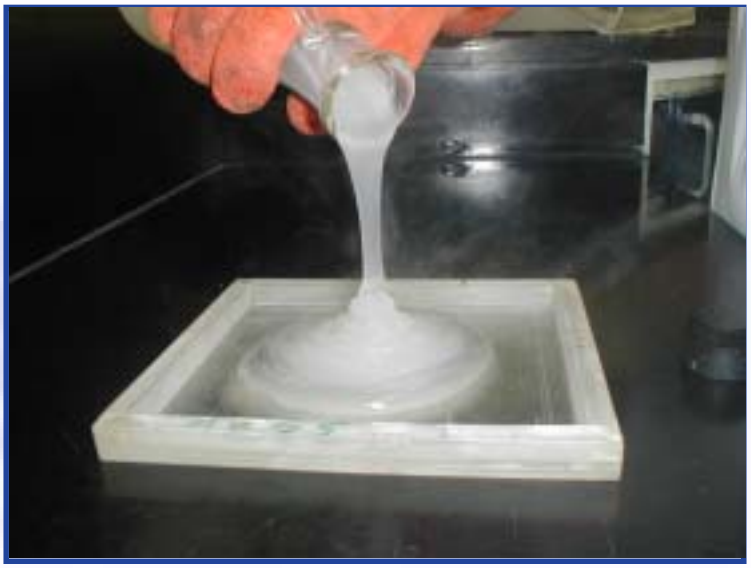
Isozymes in pictures

The following photographs illustrate the laboratory steps involved in detecting isozymes

The laboratory technician is pipetting extraction buffer onto a section of leaf. Each sample is placed in a plastic dish that is typically used to weigh chemicals. Other utensils such as glass plates can be used instead, but they must be very clean and unlikely to absorb macerated tissue.

Once the extraction buffer is placed on the leaf sample, the leaf is crushed with a plastic rod to ensure the tissue is well broken and homogenized with the buffer. This must be done as quickly as possible to prevent an increase in temperature. While the samples are being crushed, their plastic holders can rest on ice.

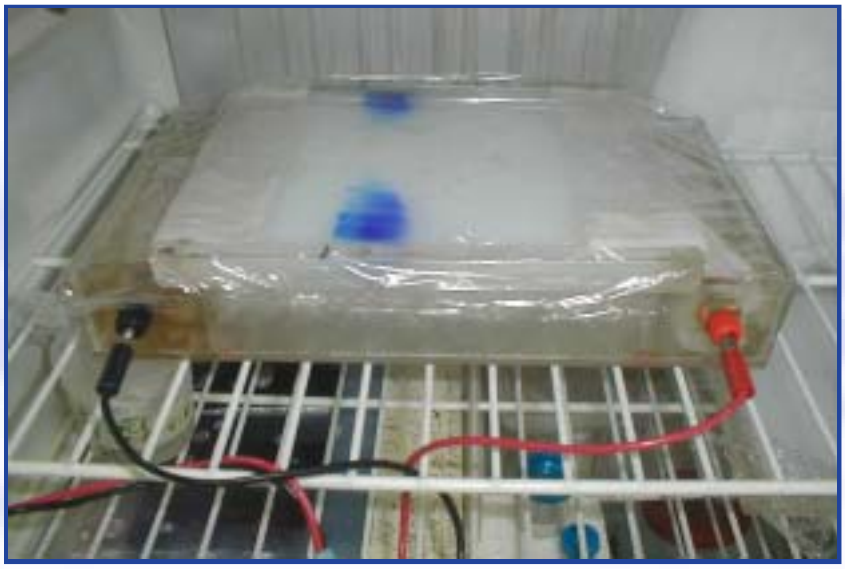
A small paper wick, cut from porous paper, is left on the homogenate, until the paper absorbs the sample.



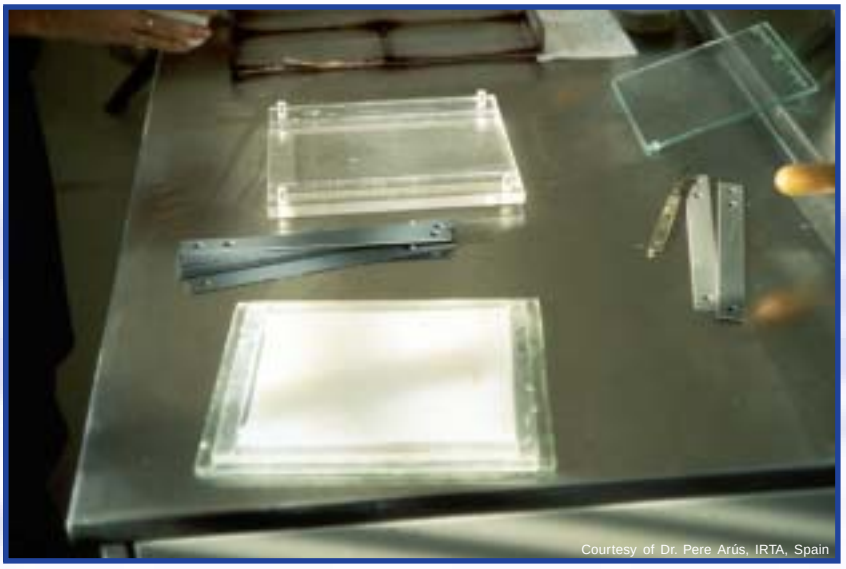
The heated starch solution is being poured into the gel mould. As the gel cools, it will become jelly-like. It will then be ready for being loaded with samples. Until used, the gel can be stored in a refrigerator.

A starch gel, set in advance, has been cut with a scalpel at about one third up the mould. Paper wicks are now being vertically placed against one of the cut sides with the help of tweezers.

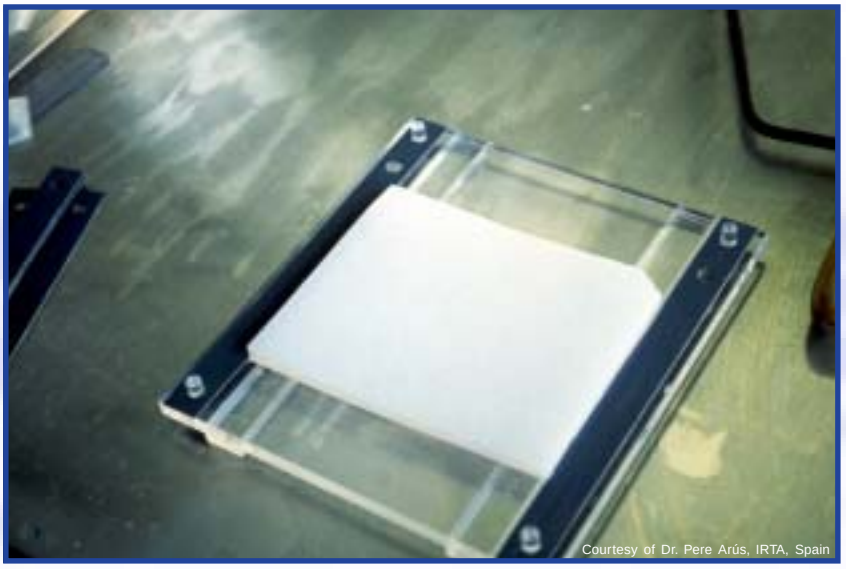
A power supply machine must be used to control the voltage and intensity of the current for electrophoresis. Many models are commercially available, and have comparable properties and capacities. In our example, two electrophoresis units can be simultaneously plugged in. A timer may also be included to halt electrophoresis at a given time.



The gel is placed on an electrophoresis unit inside a cold room or refrigerator. The unit has a buffer container at each side (cathode and anode) and contact between the buffer and gel is achieved with a sponge or piece of mesh cloth. A plastic wrap is placed on top to prevent drying out during the process and ensure good buffer transfer. Samples are loaded and their trace followed, the run being marked by a blue colorant.

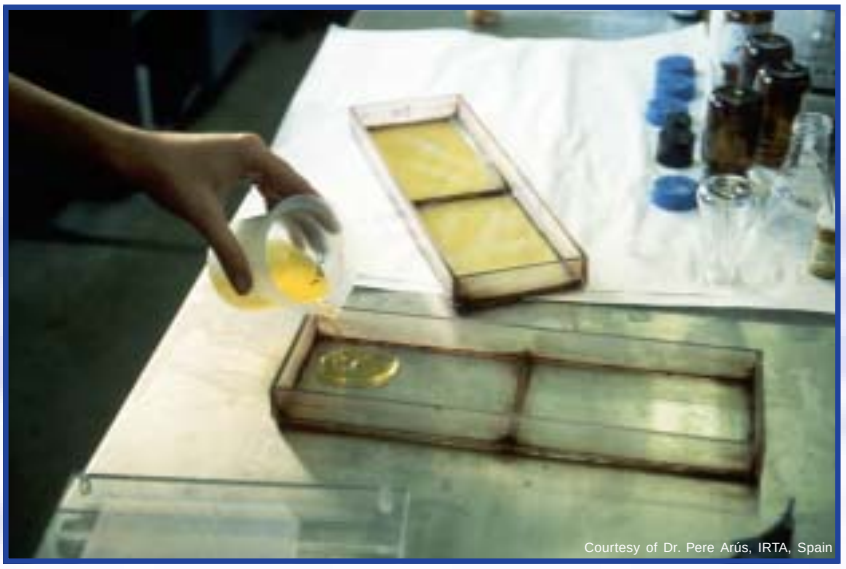


When the run is complete (usually a brownish shadow can be seen opposite the loading side of the gel), the gel must be cut into slices. The foreground shows the gel ready for cutting, with a plexiglass stand waiting for the gel's transfer (centre). A container where the slices will be placed can be seen at the photograph's top edge.



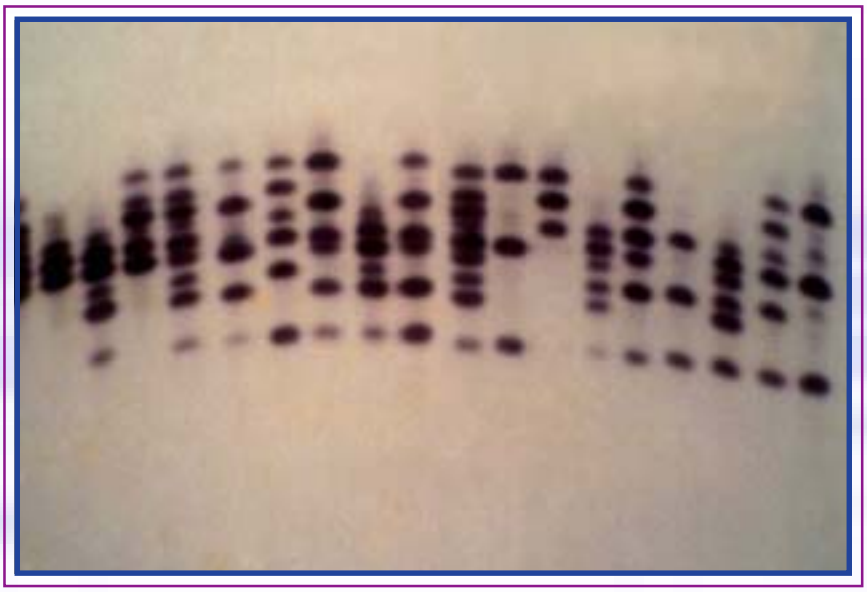
The gel is now on the stand and thin black guides are placed at each side to control the thickness of the slices. A notch is made at one corner (top right) as guide to the samples' correct location after the gel is stained.

A glass plate is placed over the gel so that pressure can be exerted while cutting, ensuring that the slices are even. A handsaw, fitted with a guitar string, is being used to cut the gel into slices.



Different staining solutions let us visualize different enzymes. Here, a yellowish staining solution is poured into a plexiglass container before a gel slice is transferred.

A slice of gel is being transferred to the container with the desired staining solution. Care must be taken on transferring the slices because their fragility. If a slice does break, the broken pieces can often be rearranged and the enzymes still visualized on the gel.



After a certain period of incubation, a stained gel slice appears similar to the photograph, which shows leaf alcohol dehydrogenase (ADH) of sainfoin (*Onobrychis viciaefolia*), a forage crop.

Interpreting banding patterns

The main issues are:

- The quaternary structure of enzymes (whether monomeric, dimeric, etc.)
- Whether the plant is homozygous or heterozygous at each gene locus
- The number of gene loci (isozymes)
- The number of alleles per locus
- How the genes are inherited

Allozymes are controlled by codominant alleles, which means that homozygotes (all alleles at a locus are similar) can be distinguished from heterozygotes (parents of the individual have contributed different alleles to that locus).

For monomeric enzymes (i.e. consisting of a single polypeptide), plants that are homozygous for a given locus will produce one band, whereas heterozygous individuals will produce two. For dimeric enzymes (i.e. consisting of two polypeptides), plants that are homozygous for that locus will produce one band, whereas heterozygous individuals will produce three because of random association of the polypeptides.

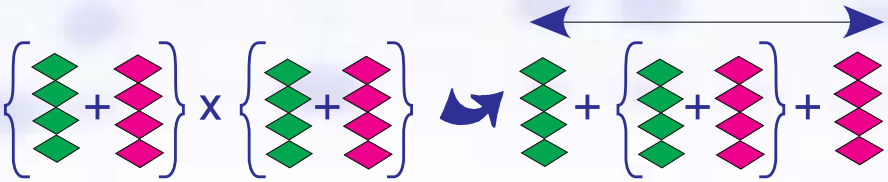
Multimeric enzymes also exist, where the polypeptides are specified by different loci. The formation of isozymic heteromers can thus considerably complicate banding patterns.

These complexities and the importance of correctly interpreting banding patterns, make genetic analysis desirable, even necessary, using progeny analysis (F_1, F_2 and back-cross) of artificial crosses between individuals with known banding patterns.

Example 1: Forming homomers

- Monomeric allozyme:
  Homozygote **F_1**

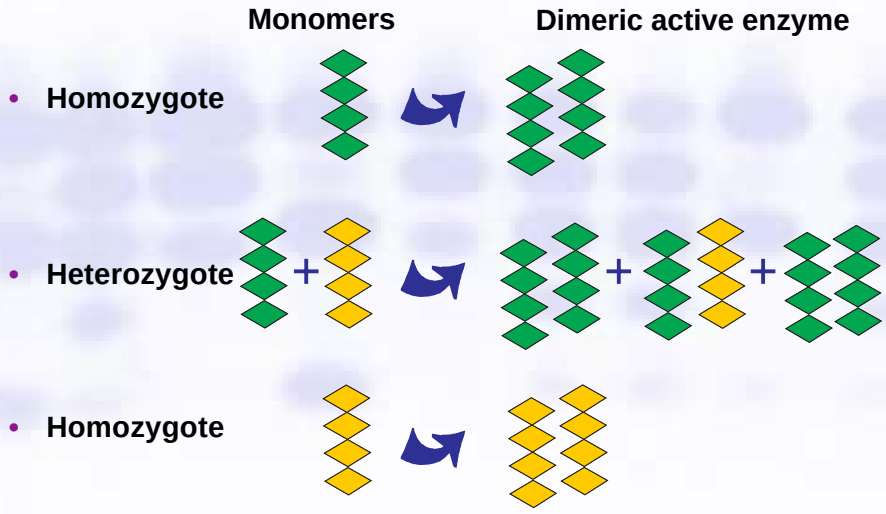
- Monomeric allozyme:
  Heterozygote **F_1**



This example shows the behaviour of monomeric enzymes in crosses. Parents are homozygous for the same allele at the same locus or heterozygotes. In the first case, all F_1 progeny will be homozygous and, as a consequence, a single band will result. If parents are heterozygous, three possible F_1 phenotypes will result.

Example 2: Forming heteromers

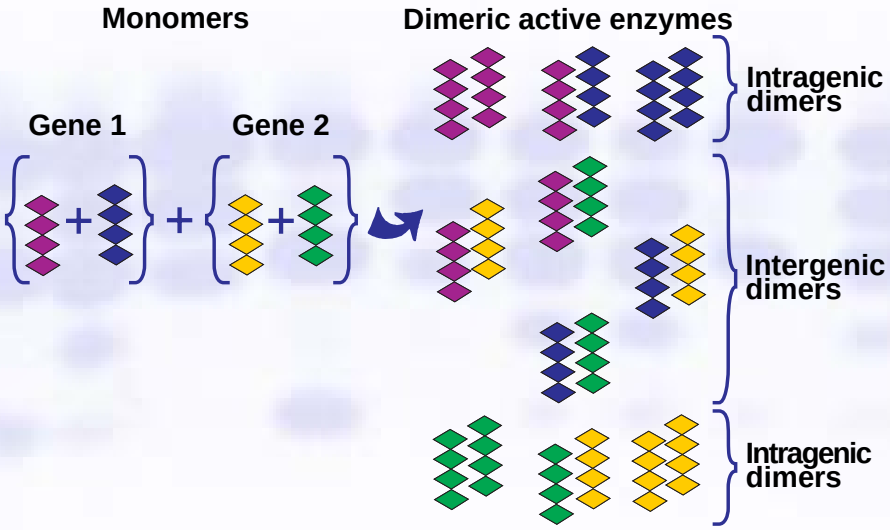
Dimeric allozyme: one gene with two alleles



In crosses, dimeric enzymes may behave in three possible ways. If both parents are homozygous for different alleles at the same locus, all progeny are heterozygous, but because of random association of the polypeptides, three combinations are possible and therefore three bands will be resolved on gel electrophoresis and staining.

Example 3: Forming heteromers (continued)

Dimeric allozyme: two genes with two alleles

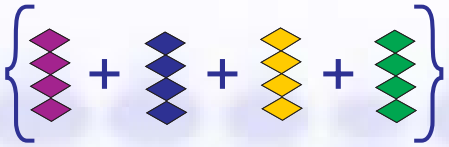


In the second example on dimeric allozymes, the enzyme's two polypeptides are coded by separate loci. Parents do not share any alleles. Random association of the polypeptides can lead to a total of 10 dimers, because both intragenic dimers (alleles at the same locus) and intergenic dimers (alleles from different loci) are formed.

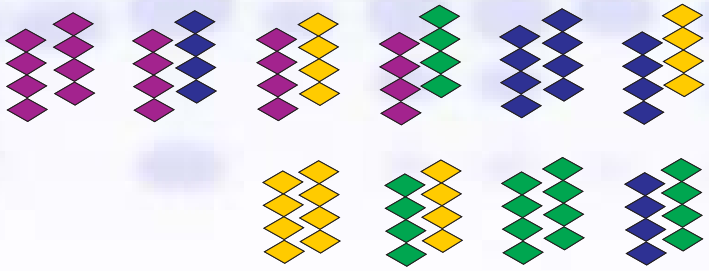
Example 4: Forming heteromers (continued)

Heterozygous tetraploid (one locus, four alleles)

- **Monomers**



- **Dimeric active enzymes**



In the example above, a heterozygous tetraploid, with four alleles at one locus, forms ten dimeric active enzymes due to random association among the four monomers.

Advantages and disadvantages

- Advantages
  - Robust and highly reproducible
  - Codominant, i.e. suitable for estimating a wide range of population genetics parameters and for genetic mapping
- Disadvantages
  - Relatively few biochemical assays available to detect enzymes
  - Phenotype-based analysis

Isozyme analysis is, in principle, a robust and reproducible method. In addition, isozymes are codominant markers and are suitable for estimating all population genetics parameters and for genetic mapping.

About 90 isozyme systems have been used for plants, with isozyme loci being mapped in many cases.

The major limitation of isozyme analysis is the low number of markers it provides, because the number of biochemical assays available to detect them is small. Consequently, the percentage of genome coverage is inadequate for a thorough study of genetic diversity.

Another disadvantage of isozyme analysis lies in the markers being based on phenotype. As such, they may be influenced by environmental factors, with differences in expression confounding the interpretation of results. Because differential expression of the genes may occur at different developmental stages or in different tissues, the same type of material must be used for all experiments.

Applications

- Gene flow and/or introgression
- Genetics of populations
- Strategies for *ex situ* conservation
- Crop evolution
- Germplasm evaluation and characterization
- Genetic erosion
- Genetic stability of conserved material

References in purple are explained in detail in the following slides.

Bartsch, D., M. Lehnen, J. Clegg, M. Pohl-Orf, I. Schuphan and N.C. Ellstrand. 1999. Impact of gene flow from cultivated beet on genetic diversity of wild sea beet populations. Mol. Ecol. 8:1733-1741.

Finkeldey, R. and O. Murillo. 1999. Contributions of subpopulations to total gene diversity. Theor. Appl. Genet. 98:664-668.

Ibáñez, O., C. Calero, M. Mayol and A. Rosello. 1999. Isozyme uniformity in a wild extinct insular plant, *Lysimachia minoricensis* J.J. Rodr. (Primulaceae). Mol. Ecol. 8:813-817.

Ledig, F.T. 2000. Founder effects and the genetic structure of Coulter pine. J. Hered. 91(4):307-315.

Li, Z. and J.N. Rutger. 2000. Geographic distribution and multilocus organization of isozyme variation of rice (*Oryza sativa* L.). Theor. Appl. Genet. 101:379-387.

Liu, F., R. Von Bothmer and B. Salomon. 1999. Genetic diversity among East Asian accessions of the barley core collection as revealed by six isozyme loci. Theor. Appl. Genet. 98:1226-1233.

Maquet, A., I. Zoro Bi, M. Delvaux, B. Wathelet and J.P. Baudoin. 1997. Genetic structure of a Lima bean base collection using allozyme markers. Theor. Appl. Genet. 95:980-991.

Ortiz, R. and Z. Huaman. 2001. Allozyme polymorphisms in tetraploid potato gene pools and the effect on human selection. Theor. Appl. Genet. 103:792-796.

Rouamba, A., M. Sandmeier, A. Sarr and A. Ricroch. 2001. Allozyme variation within and among populations of onion (*Allium cepa* L.) from West Africa. Theor. Appl. Genet. 103:855-861.

Shapcott, A. 1998. The patterns of genetic diversity in *Carpentaria acuminata* (Arecaceae) and rainforest history in northern Australia. Mol. Ecol. 7:833-847.

Example: *Lysimachia* sp.

- Title:

  Isozyme uniformity in a wild extinct insular plant, *Lysimachia minoricensis* J.J. Rodr. (Primulaceae).

  Mol. Ecol. 1999. 8: 813-817

- Objective:

  To evaluate the genetic diversity of seed accessions of *Lysimachia minoricensis* conserved and provided by 10 European botanical gardens

- Materials and methods:

  A total of 158 plants were analysed for 13 enzymes (22 loci)

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Example: *Lysimachia* sp. (continued)

- Results:

  No electrophoretic variation was detected for any of the enzymes assayed (22 loci)

- Discussion on lack of variation:
  - Electrophoretic techniques resolve only a small portion of genetic variation
  - Low sample size? The sample offers a 95.6% probability of detecting any variant allele that existed at an overall frequency of at least 1%
  - Is this lack of detectable variation unexpected? Results address more the question of the amount of genetic variation preserved *ex situ* than what the level was before extinction

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Example: *Lysimachia* sp. (continued)

Conclusions:

The exchange system among botanical gardens is not adequate for effective conservation if the genetic variation within a species is underrepresented in the plant collections

Example: Lima bean

- Title:
  Genetic structure of a Lima bean base collection using allozyme markers. Theor. Appl. Genet 1997. 95:980-991
- Objective:
  To evaluate genetic diversity and structure within a lima bean (*Phaseolus lunatus* L.) base collection, involving several, widely distributed, wild accessions and landraces
- Materials and methods:
  Ten enzyme systems were used to analyse 235 lima bean accessions (1-5 seeds each) collected in Latin America and the Caribbean

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Example: Lima bean (continued)

Results and discussion:

- Thirteen loci (32 alleles) were found for 10 enzyme systems
- Specific alleles were identified in each gene pool. The dendrogram clearly showed two main clusters:
  - Accessions from the Andes with an Andean seed-protein pattern
  - Mesoamerican and Andean accessions with a Mesoamerican seed-protein pattern
- Both Andean and Mesoamerican landraces were grouped with their respective relatives
- On average, the lima bean showed 76% and 24% of the total diversity, respectively, among and within accessions. Reasons are selfing, occurrence of small populations, and low gene flow

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Example: Lima bean (continued)

Conclusions:

- A conservation programme of *P. lunatus* must include wild and cultivated forms from both gene pools
- As the genetic diversity is distributed mainly among accessions, more populations or accessions should be preserved to ensure the retention of allelic and genotypic diversity for both gene pools and botanical forms

Example: Onion

- Title:

  Allozyme variation within and among populations of onion (*Allium cepa* L.) from West Africa. Theor. Appl. Genet. 2001. 103: 855-861

- Objective:

  To evaluate genetic diversity in local populations of onion, using isozymes

- Materials and methods:
  - Sixteen local cultivated populations sampled from five countries of West Africa were studied
  - Nine enzyme systems were assayed

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Example: Onion (continued)

Results:

- Four systems were polymorphic (ADH, MDH, 6-PGDH, PGI), with a total of nine alleles
- The mean number of alleles found per polymorphic locus was 2.25. In addition, 66.6% of alleles were present in all populations
- Allele *adh-a2* was absent in the five populations originating from Burkina Faso. Allele *6-pgdh-a2* was present only in two populations, one from southern Niger and the other from northern Nigeria

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Example: Onion (continued)

- Discussion:
  - Weak geographical differentiation between populations may have resulted from commercial exchange between countries of the same language
  - Overall, heterozygotes were few, maybe because of autogamy in the populations sampled or genetic drift resulting from seed production involving tiny quantities of bulbs as progenitors
- Conclusions:

  Similar studies on a continental scale over Africa would be advisable to better understand the species's variability

In summary

- Isozyme technology is based on protein extraction, separation by gel electrophoresis and histo-chemical staining
- The equipment needed is simple
- The interpretation of banding patterns is typically codominant and, because of its complexity, may require genetic analysis
- Isozymes as genetic markers are highly reproducible

By now you should know

- Main steps involved in isozyme technology
- Main points for consideration when interpreting banding patterns
- Advantages and disadvantages of isozymes as genetic markers for diversity studies

Basic references

Hamrick, J.L. and M.J. Godt. 1989. Allozyme diversity in plant species. Pp. 43-63 in Plant Population Genetics, Breeding, and Genetic Resources (A.H.D. Brown, M.T. Cleg, A.L. Kahler and B.S. Weir, eds.). Sinauer Associates, Sunderland, MA.

Manchenko, G.P. 1994. Handbook of detection of enzymes on electrophoretic gels. CRC Press, Boca Raton, FL.

Murphy, R.W., J.W. Sites, D.G. Buth and C.H. Haufler. 1996. Proteins: isozyme electrophoresis. Pp. 51-20 in Molecular Systematics (2nd edn.). (D.M. Hillis, C. Moritz and B.K. Mable, eds.). Sinauer Associates, Sunderland, MA.

Soltis, D.E. and P. Soltis (eds.). 1989. Isozymes in plant biology. Dioscorides Press, Portland, OR.

Tanksley, S.D. and T.J. Orton (eds.). 1983. Isozymes in plant genetics and breeding, parts A and B. Elsevier Science, Amsterdam, Netherlands.

Next

DNA-based technologies

DNA basics

- DNA-based technologies
  - Restriction fragment length polymorphisms (RFLPs)
  - PCR-based technologies
- Complementary technologies
- Final considerations
- Glossary