

## Electrophoresis Studies of *Ranunculus triplodontus* Populations

SURANTO

Biology Department, Faculty of Mathematics and Sciences, Sebelas Maret University Surakarta.

Received: June 5, 2001; Accepted: July 31, 2001

### ABSTRACT

The main purposes of this research were to investigate whether the two distinct types of the morphological character of *Ranunculus triplodontus* were genetically controlled or environmental influence. In order to prove the above, electrophoretic examinations were carried out employing for four enzyme systems. The medium support of polyacrilamide was chosen. The samples were collected from seven populations around central plateau and the leaves were used as the isozyme data. The result indicated that variation occurred in certain populations. However, this isozyme data were not able to separate the two types of *R. triplodontus* into different species. Based on the cluster analysis showed that three groups of seven populations of *R. triplodontus* were appeared. This research confirms that morphologically distinct species was not supported by the isozyme data, thus the variation found in certain population was mainly influenced by the environmental conditions, and therefore could not be considered as taxonomically distinct.

© 2001 Jurusan Biologi FMIPA UNS Surakarta

**Key words:** *Ranunculus triplodontus*, isozyme.

### INTRODUCTION

Morphologically, *R. triplodontus* possesses variable characters not only in the leaves but also in the flowers. The species can be divided, into two distinct types. The first type has dissected leaves and the flowers have 5 petals. The second type has simpler tridentate, cuneate leaves, and the flowers are 2-3 petalled or apetalous. Both types remained unchanged for leaf and flower morphology during transplant trials (Backer and Bakhuizen van den Brink, 1963; Bentham, 1864). It is a little surprising that past workers in *Ranunculus* have not proposed taxonomic separation of these forms into distinct species or at least sub-species. Menadue (1986) hinted at the possibility of reclassification but opted to wait for further investigations to be carried out. Results of transplant observations, as reported above, indicate that the first type (rare one) maintained its differences from the second type (common one). This suggested that these differences were real genetic differences between the two forms. Accordingly,

electrophoretic examinations of both were conducted to investigate whether their isozyme patterns showed parallel differences.

### MATERIALS AND METHODS

Plants from seven populations around the Central Plateau were examined electrophoretically. The Liawenee population represented the rare form and the other 6 populations the common form. The collected plants was identified with manuals of Bentham and Hooker (1865), Candole (1818-1821), Curtis (1956; 1967), Curtis and Morris (1975), Hooker (1982), and Menadue and Crowden (1989).

Electrophoresis procedures used were further explained in the next pages. Table 1 gives the location sites and the number of plants used in this study, while a map of the collection sites is shown in Figure 1.

#### *Gel preparation*

In order to make the best quality of polyacrilamide gel; both two kind of stock

solutions were prepared. Diluting 4,5 grams of TRI (hydroxymethyl) made *stock solution A*. methylamine (PURISS) and 0,51 grams of citric acid into 500 ml deionized water, while the *stock solution B* was prepared by mixing the 30 grams of Acrylamide and 0,80 grams of NN-methylene-bis-acrylamide into 100 ml of deionized water.

**Tabel 1.** Population sites and plant number of *R. triplodontus* used in this study.

Sites	Abbreviation	Plant Number	Annotations
Liawenee	Lwn	16	V
Nive River	NRv	20	V
Rats Castle	RCs	16	V
Projection Bluff	Pbf	15	V
Clarence Weir	Cwr	20	V
Ouse River	ORv	13	V
Wild Dog Plains	WDP	12	+

Annotations:

- V: indicates the plant samples used both for transplant and electrophoresis,
- +: only for electrophoresis

#### *Casting the gel*

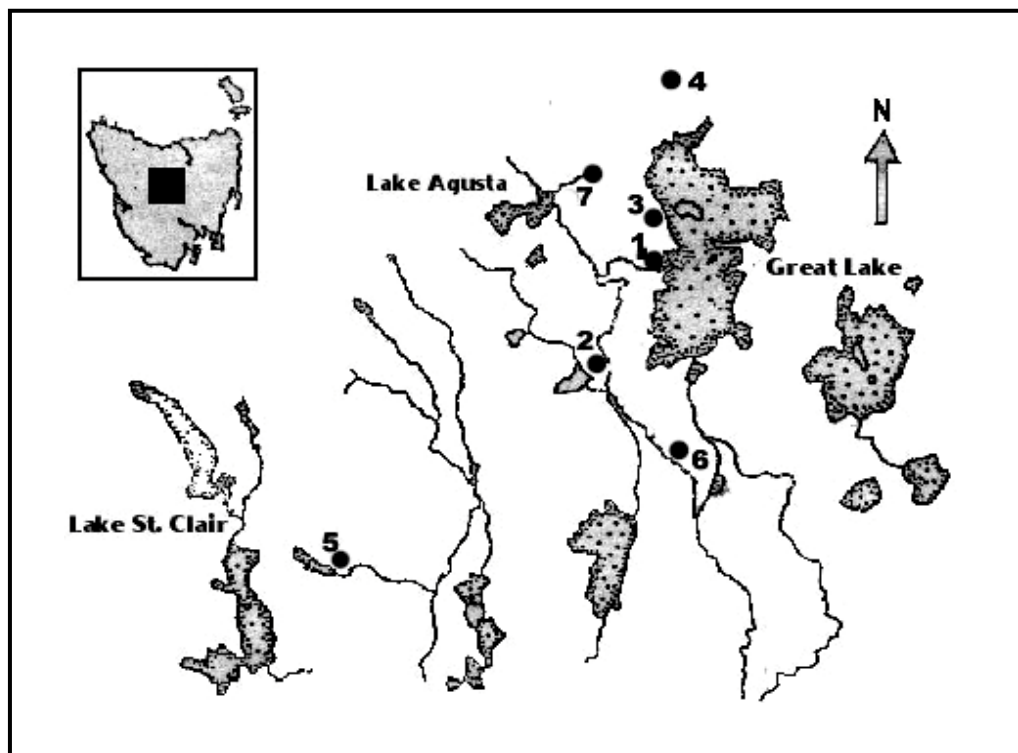
The gel was made by mixing 20 ml of solution B and 40 ml of solution A. This mixture was deaerated on a Buchi rotary evaporator for 5 minutes after which 0,04 ml of N,N,N',N'-tetramethyl-ethylenediamine was added and with carefully mixed. To polymerise the gel, 0,06 grams of ammonium persulphate was added and mixed carefully immediately before pouring the solution into the gel mould (BIO-RAD Model 361). Using this model, at least 4 thin gels each with 10-14 slots can be cast simultaneously.

#### *Protein extracting solution*

The solution of protein extraction was made up by diluting 0,018 grams of cysteine, 0,021 grams of ascorbic acid and 5 grams of sucrose into 20 ml of borax buffer pH 8,4.

#### *Extraction and loading the samples*

Laminas and petioles were examined separately. Material from each plant was ground individually in a staining dish using 0.15-0.35 ml of protein extracting solution for laminas and 0,1-0.15 ml for petioles. Despite



**Figure 1.** Sites of sample collections of *R. triplodontus* in Central Plateau (black block). Annotations: 1. Liawenee, 2. Nive River, 3. Rats Castle, 4. Projection Bluff, 5. Clarence Weir, 6. Ouse River, 7. Wild Dog Plains.

the voluminous literature on extraction methodology which suggests the need to use frozen plant material (liquid nitrogen), it was found unnecessary for the systems studied in this project to use other than an ice cool buffer and hold plant material and extracts in a ice bath. The extracts were transferred to a small glass vial, 2 mm diameter, 3 cm long, and centrifuged at 3500 rpm for 15 minutes. The supernatants were then applied in the gel slots. The amount of sample loaded in each slot was, for peroxidases about 10-15 ul, while for the other enzyme about 15-24 ul.

### *Electrophoresis*

The electrophoresis chamber used in this project was a mini vertical slab cell manufactured by BIO-RAD, USA, model 360. This model has advantages in allowing use of very small amounts of samples, as well as allowing a short running time.

Electrophoresis was conducted at a constant current of 5 mA for peroxidase (PER) and 7 mA for esterase (EST), malate dehydrogenase (MDH), and acid phosphatase (ACP), at room temperature for about 60 minutes including a pre-electrophoresis time of approximately 10 minutes. It was stopped when the bromophenol blue marker dye had travelled about 56 mm from the slot toward the anode.

### *Staining procedures:*

Four enzyme stains were used routinely.

1. *Peroxidase* (PER) was prepared by diluting 0.0125 grams of O-dianisidine into 25 ml of acetone. Then 50 ml of 0.2 M acetate buffer pH 4.5 was added and 2 drops of H<sub>2</sub>O<sub>2</sub> lastly given.
2. *Esterase* (EST) was prepared by dissolving 0.0125 grams of a-naphthyl acetate in 2.5 ml acetone. After that 50 ml of 0.2 M phosphate buffer pH 6.5 and 0.0125 grams of Fast Blue BB Salt were added.
3. *Malate dehydrogenase* (MDH) was made up by mixing 15 ml of 0.1 M Tris-HCl pH 8 and 0.020 grams of MTT (2,5-Diethylphenyl tetrazolium Bromide) and 0.005 grams of PMS (Phenazine-Methosulfate) into 125 ml of deionized water. Mixed them gently and then 10 ml of 0.2 M. Sodium malate pH 7.5 was lastly added. The gel was incubated for 30-40 minutes in the dark. A fresh solution containing 0.020 grams of NAD (nicotinamide adine dinucleotide) was used to transfer the gel.

4. *Acid phosphatase* (ACP) was made by diluting 0.0125 grams of a-naphthyl phosphate into 2.5 ml of acetone and then 75 ml of 0.2 M acetate byffer pH 4.5. 0.025 grams of Fas Beach K Salt and 0.025 grams were gently mixed.

All staining procedures in this experiment were conducted at room temperature. For peroxidase and esterase stains refer to Mills and Crowden (1968), for malate dehydrogenase stains refer to Brown *et al* (1978), and for acid phosphatase stains refer to Adam and Jolly (1980).

### *Cluster analysis*

Data used in this cluster analysis were isozyme band numbers. The bands were treated as characters, by giving values of 1 and 0 to indicate presence (i.e. detectable) and absence (i.e. not detectable) of bands, respectively (Sneath and Sokal, 1973).

There were 31 enzymic characters, 7 isozyme bands of peroxidase, 9 of esterase, 6 of malate dehydrogenase and 9 of acid phosphatase. A total of 341 individual plants belonging to the 11 species from a number of populations around the Central Plateau (table. 8) were scored with respect of 31 enzymic characters, 7 isozyme bands of peroxidase, 9 of esterase, 6 of malate dehydrogenase, and 9 of acid phosphatase.

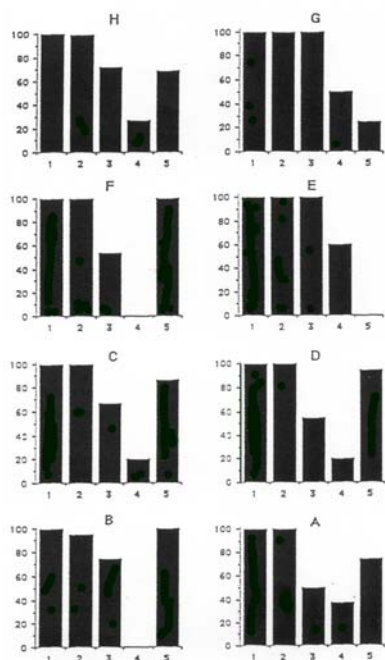
The data were then computed using the SAS program. The clustering strategy was Average linkage Ouster Analysis using Squared Euclidean Distance (UPGMA).

## **RESULTS AND DISCUSSION**

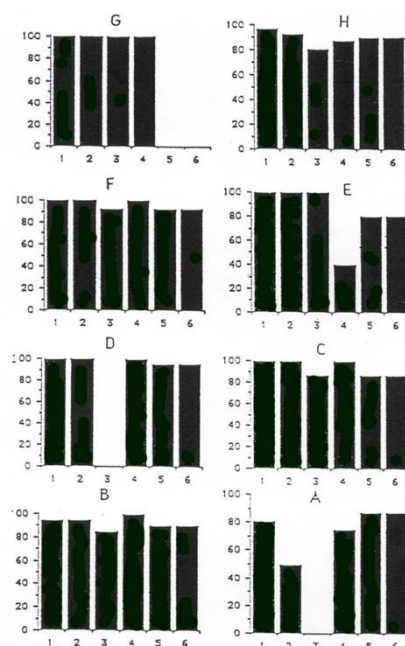
Figure 2-5 shows the patterns of isozyme bands for each of the *R. triplodontus*, populations A, B, C, D, E, F, G and H for all populations. Detailed band frequency of each population is reported below.

### *Bands pattern*

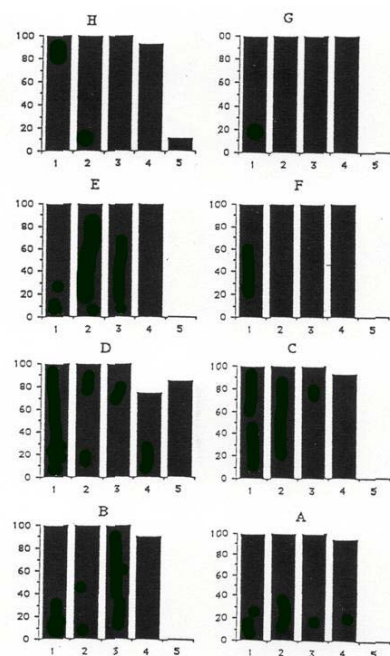
**Peroxidase isozymes:** Bands 1 and 2 were present in all populations. Band 3 was present in all, but varied in frequency in different populations, ranging from 50-60 %. Band 4 was absent from Nive River and Projection Bluff, but present in quite varied frequencies in the other five populations (20-60 %). Band 5 was absent in Projection Bluff (Figure 2).



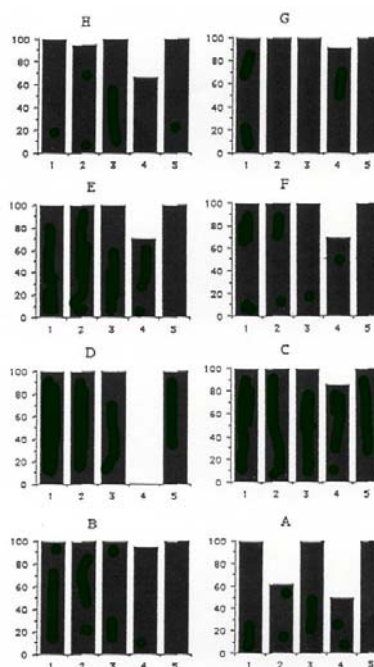
**Figure 2.** Peroxidase isozyme pattern of each population of *R. triplodontus*: A. Liawenee, B. Nive River, C. Rats Castle, D. Projection Buff, E. Clarence Weir, F. Ouse River, G. Wild Dog Plains, H. All populations. The X-axis indicates the band number and the Y-axis indicates the frequency of bands present.



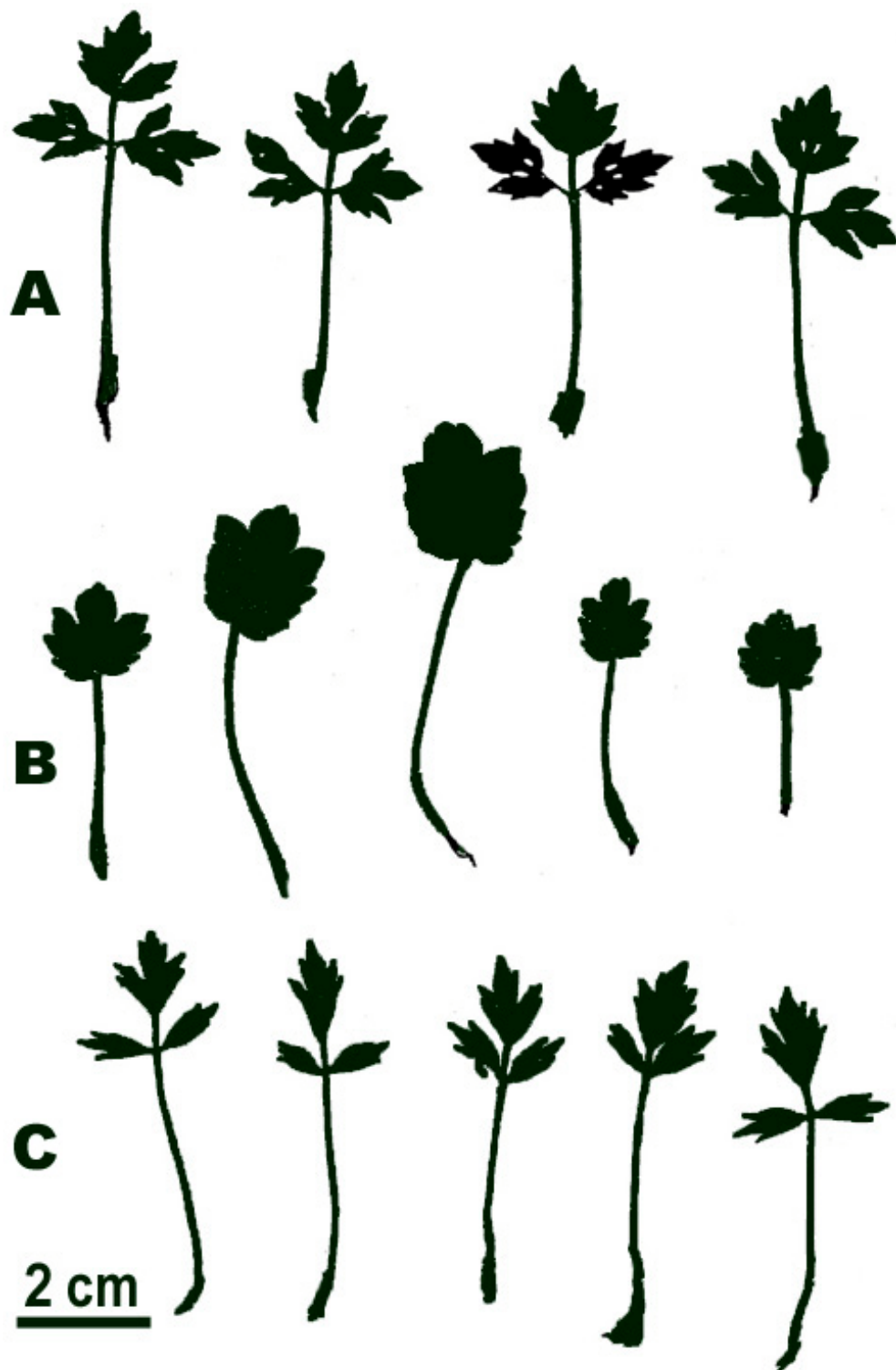
**Figure 4.** Malate dehydrogenase isozyme pattern of each population of *R. triplodontus*: A. Liawenee, B. Nive River, C. Rats Castle, D. Projection Buff, E. Clarence Weir, F. Ouse River, G. Wild Dog Plains, H. All populations. The X-axis indicates the band number and the Y-axis indicates the frequency of bands present.



**Figure 3.** Esterase isozyme pattern of each population of *R. triplodontus*: A. Liawenee, B. Nive River, C. Rats Castle, D. Projection Buff, E. Clarence Weir, F. Ouse River, G. Wild Dog Plains, H. All populations. The X-axis indicates the band number and the Y-axis indicates the frequency of bands present.



**Figure 5.** Acid Phosphatase isozyme pattern of each population of *R. triplodontus*: A. Liawenee, B. Nive River, C. Rats Castle, D. Projection Buff, E. Clarence Weir, F. Ouse River, G. Wild Dog Plains, H. All populations. The X-axis indicates the band number and the Y-axis indicates the frequency of bands present.



**Figure 2.** Three sort of mature leaves *R. triplodontus* collected from Liawenee populations. Lives of A and C will quite distinctive compared with B, which is the common type for most population sampled.

**Esterase Isozymes:** Bands 1-4 were present in all populations with quite high frequencies. Band 5 was present only in the Clarence Weir populations (Figure 3).

**Malate Dehydrogenase Isozymes:** Band 4 varied in frequencies ranging 30-100 %. Band 3 was absent from Liawenee, and Clarence Weir. Bands 5 and 6 were absent from Wild

Dog Plains. Bands 1 and 2 were present in all populations, as was band 4 (Figure 4).

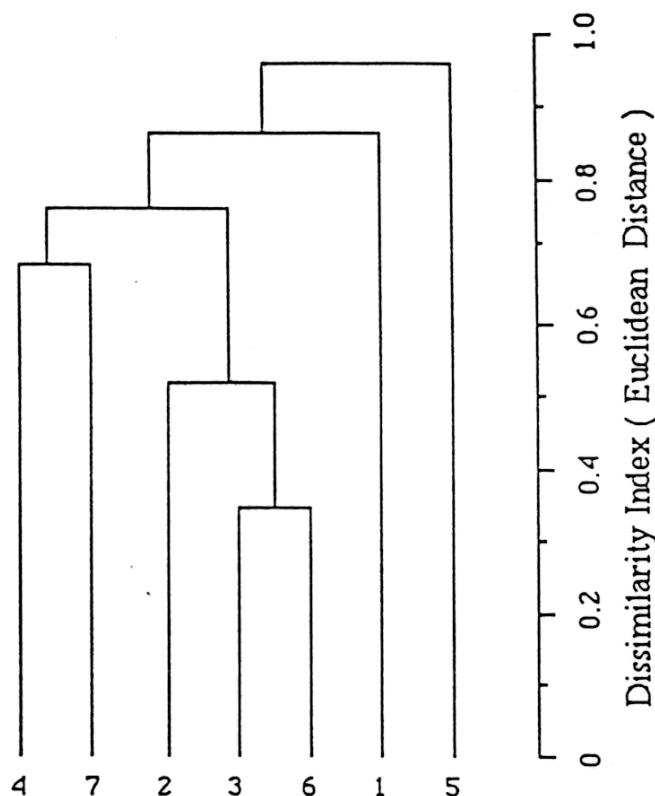
**Acid Phosphatase Isozymes:** Band 4 was absent only from Clarence Weir but varied in the frequency of each population, ranging 50-95 %. Band 1, 2, 3, and 5 were present in all populations (Figure 5).

#### *Morphological variations*

Figure 2 shows some variations of mature leaves *R. triplodontus*. Comparison of leaf morphology of this species showed those two distinct types. The Liawenee population as shown in Figure 2, A and C were quite different compared with the B type. The common type of leaf morphology of *R. triplodontus* as illustrated in Figure 2, B was also found in other populations for Ouse River, Clarence Weir, Nive River and Rats Castle. Different

environmental pressures in the natural habitats have resulted in populations with morphological variations, which appear to have stabilised genetically. The studies with *R. triplodontus* indicate that polymorphism shown by this did not reveal a "plasticity" component on transplanting, so that it is essentially of genetic nature. However of the enzyme systems used in this study, only malate dehydrogenase gave a quantitative differentiation of the Liawenee type. More basic studies with alternative isozyme assays may be required, before a better result obtained.

The Liawenee plants were sampled from a different habitat compared to the other population. They grew in the small canals with a rocky floor and edge. A number of plants were taken from the stream with most of the leaves submerged.



**Figure 6.** Malate dehydrogenase isozyme pattern of each population of *R. triplodontus*: A. Liawenee, B. Nive River, C. Rats Castle, D. Projection Bluff, E. Clarence Weir, F. Ouse River, G. Wild Dog Plains, and H. All populations. The X-axis indicates the band number and the Y-axis indicates the frequency of bands present.

For the two populations Projection Bluff and Wild Dog Plains, which clustered together, the habitats were quite similar with the plants growing in the muddy flowing water. The three populations grouped in the major cluster, Rats Castle, Nive River and Ouse River, attitudinally were in between Projection Bluff and Clarence Weir. These three populations had a similar habitat where the plant grew in the moist soils associated with tussock grasses though some times flooded by the water. Clarence Weir environmental conditions were closer to the Liawenee's environment. These plants grew in the moist soil amongst rocks near to the lagoon, and occasionally flooded by water.

#### *Dendrogram*

The significant result of this study is that the Liawenee population (the rare form) is not separated from the other populations of *R. triplodontus* sampled.

A cluster analysis was conducted using squared Euclidean distance. Results in Figure 6. The groupings presented by the dendrogram indicate some genetic diversity in this species. Group one, Projection Bluff and Wild Dog Plains relates the populations of highest altitude. Group two, contains 3 middle altitude populations (Nive River, Castle and Ouse River). The Liawenee populations stand alone, as does the low altitude population, Clarence Weir. However there is no convincing data to support the hypothesis that the Liawenee population is taxonomically distinct.

### CONCLUSION

Based on the genetic data (isozymes) several populations of *R. triplodontus* that showed varied in rather than environmentally

influences, their morphological population were not genetically based.

### REFERENCE

- Adam and Jolly. 1980. Electrophoretic Buffer System and Stain Recipes. *In* CSIRO, Division of Forest Research (Ed.) 1982. pp.: 68-80.
- Backer, C.A. and R.J. Bakhuizen van den Brink (1963). *Flora of Java*. Volume I. Groningen: Noordhoff.
- Bentham, G. 1864. *Flora Australensis*. Volume 2. London: Reive Br Co.
- Bentham, G and J.D. Hooker. 1865. *Genera Plantarum*. Volume I. London: Reive Br Co.
- Brown, A.D.H., E. Nevo, D. Iohary, and O. Dagon. 1978. Genetic variation in natural populations of wild barley (*Hordeum spontaneum*). *Genetica* 49 (2/3): 97-108.
- Candole, A.P. de. 1818-1821. *Regni Vegetabilis Systema Naturale*. Two Volumes. Paris: Treuttel et Wurtz.
- Curtis, W.M. 1956. *A Student's Flora of Tasmania*. Part I. Hobart: Government Printing.
- Curtis, W.M. 1967. *Introduction in the Endemic Flora of Tasmania*. London: Airel Press.
- Curtis, W.M. and D.J. Morris. 1975. *A Student's Flora of Tasmania I*. 2<sup>nd</sup> edition. Hobart: Government Printing.
- Hooker, J.D. 1882. *The Flora of British India* Volume I. Ranunculaceae to Sapindaceae. New Delhi: Bishen Sing Mahendra PA Sing Pub.
- Mills, A.K. and R.K. Crowden. 1968. Distribution of soluble proteins and enzyme during early development of *Pisum sativum*. *Aust J. Biol. Sci.* 21: 1131-1141.
- Menadue, Y. 1986. Taxonomy of genus *Ranunculus* in Tasmania. *Ph.D. Thesis*. Tasmania: New Castle University.
- Menadue, Y and R.K. Crowden. 1989. Tasmanian species of *Ranunculus*. *A New Key Paper and Proceedings of the Royal Society of Tasmania*. Volume 123: 87-96.
- Sneath, P.H.A and R.R. Sokal. 1973. *Numerical Taxonomy*. San Francisco: W.H. Freeman and Co.