GENETIC DIVERSITY OF GENUS *Eulophia* (ORCHIDACEAE) IN SELECTED WETLANDS OF UGANDA BASED ON AFLP ANALYSIS

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AFLP

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**ABSTRACT**

Present study involving screening of genetic diversity within and among populations of *Eulophia* was carried out using Amplified Fragment Length Polymorphisms (AFLPs). Cluster analysis of all the AFLP data by unweighted pair group method of arithmetic averages (UPGMA) resulted in a dendrogram that very closely conformed to the difference in populations. The dendrogram shows that similar species were positioned close to one another but individuals there in are clearly genetically different both within the sites and between sites. The significant between population variance probably indicates restricted gene flow. Clusters shown in UPGMA dendrogram were consistent with the results of principal coordinate ordinance (PCO) using NTSYS. In conclusion, despite the small number of samples analyzed at the selected sites, they are genetically varied at the sites and among populations.
1 Introduction

In Africa, wetlands cover about 1.2 million km² while in Uganda, wetlands cover about 12% of its 241,500 km² total area. Of this, 3.2% is permanent swamp while 15.1% is open water (National Environment Action Plan Secretariat, 1995). The nature of inland water ecosystems promotes very high levels of genetic diversity (within species, of varieties, strain, and populations). This is often because groups of freshwater organisms can be isolated from one another between, and even within, catchments, including geographically very close or even adjacent ones (UNDP, 2000). Leviticus (2001) argues that environmental factors influence the genetic composition of populations and directly influence the phenotypic variation. The molecular genetics approaches are particularly useful for identifying the variations that exist within species. These genetic relationships among populations are often used to find out the patterns of variation, movement and the degree of gene flow among populations (Avise, 1994). Milligan et al. (1994) further explains that continuous inbreeding and genetic drift are the two important possible reasons of loss in genetic variability. In this regard the case with Eulophias in Uganda’s wetlands is limited.

Variations may occur in a population which is present in small areas. This is especially common with the self-fertilizing plant species. Several distinct genotypes adapted to take advantage of the prevailing conditions may occur within a population covering only a few metres. Plants can also exhibit significant between population genetic diversity, especially within a widespread species. In asexual populations, the absence of segregation and recombination is predicted to lead to reduced levels of genetic variation (Silander, 1985) but genotypes preserve their genetic integrity.

Genetic diversity in a population can be measured by the number of distinct alleles per locus. In maintenance of allelic variation, bottlenecks and genetic drift have naturally strong effects on the genetic composition of a population both in quantitative (number of alleles) and qualitative (genetic variance) sense. However, the immediate effects on allele’s variation are far most significant than those on the genetic variance. Drift however can have serious influence on allele frequencies as well as on genetic variance (Frankel & Soule, 1981). Genetic differences between populations, particularly those of long lived perennials, are usually a result of a long term evolution. The locally common alleles are critical since the variation that makes populations adapted to their environment. As reported by Milligan et al., 1994, populations poorly adapted to the natural environment are prone to different environmental bottlenecks and drift.

Various studies of genetic variations within terrestrial orchids like Cymbidium goeringii (Chung et al., 1998), Caladenia tentaculata (Peakall & Beattie, 1996), Cephalanthera longibracteata (Chung et al., 2004), Oriental Cymbidium (Choi et al., 2006), Dendrodium officinale (Xuexia Li et al., 2007) and Sun (1997) have been carried out. Such genetic information plays an important role in the development of conservation biology by focusing attention on the evolutionary properties and population genetics of small populations. The importance of genetic diversity for maintenance of viable populations in the face of a changing environment need not be over emphasized.

Genetic diversity amongst individuals allows for occupying of differing microhabitats and broadens tolerance ranges of a population beyond the range of an individual (Huennke, 1991). The available genetic variation determines a population’s response to selection pressures imposed by future changes in the environmental conditions. This affects the probability of population and species persistence (Crow, 1992). Similarly, Murray & Young (2001) reported that the level of variation detected reflects future adaptation or individual fitness. Using the AFLPs, pattern of similarity among genotypic classes for example in number of shared nucleotides reflects the genealogical similarity of those classes and those haplotypes that share a larger number of nucleotides are known to have a common ancestor more recently than those that share a smaller number. The genetic structure of subdivided populations was readily observable in patterns of marker variation.

From study carried out on the effects of fragmentation on genetic variation in Platanthera leucophaea (Orchidaceae) by Wallace (2002), it was reported that genetic and geographic distances were not significantly correlated and it suggested lack of inter-population gene flow and/or genetic drift within populations.

Similarly, Travis et al. (1996) assessed genetic diversity within and among populations of Astragalus crenophylax, using AFLP. They reported a total of 220 polymorphic fragments scored using nine primer combinations. They measured diversity within each population and subpopulation on the basis of average heterozygosity and the proportion of polymorphic genes. Genetic diversity within populations was directly affected by population size, with the largest population having the greatest diversity. Comparisons of AFLP patterns revealed greater similarity among plants collected from the same population than among plants collected from different populations. Similarly, Genetic relationships among 12 soybean (Glycine max) genotypes were determined by Powell et al. (1996) using RFLP; RAPD; AFLP and microsatellite markers. The AFLP assay was determined to have the highest marker index value, indicating its superiority in detecting polymorphism in soybean genotypes. Use of AFLP to detect genetic variation among 32 genotypes of tea (Camellia sinensis) was employed by Paul et al. (1997). The principal coordinate analysis of this study showed common ancestry of Kenya and India tea. Calculations on genetic diversity revealed that 79% was detected within populations and 21% between Indian and Kenyan populations. Estimates of diversity within populations revealed that the China type tea was the most critical.
variable and the Cambodian population was the least variable. Xuexia Li et al. (2007) also used AFLP to study genetic diversity and conservation of twelve populations of the endangered Chinese *Dendrobium officinale*.

Sun & Wong (2001) studied the genetic structure of three orchid species one of which was *Eulophia sinensis*, an out crossing colonizer. The results showed substantial genetic variation within populations of *E. sinensis*. They also report that genetic diversity at the species level occurred between populations in all the three species despite the breeding system. The pattern of genetic variation was related to their differences in breeding system and colonizing ability. Similarly, Gustafsson, 2000 reports on the genetic variation in the fragrant orchid, *Gymnadenia conopsea* described at the level of microsatellite variation within and among 10 Swedish populations. It was reported that high genetic variation within and low genetic divergence among populations occurred. There was also a close to significant correlation between population size and number of alleles at the 95% level.

Although the Orchidaceae constitute up to 10% of all flowering plant species (Dressler, 1981), few reports are available on studies of population genetic diversity in wild orchids using the different markers. *Eulophia*, an Orchidaceae, commonly grows in seasonal and permanently wet areas. However, due to the ever increasing human population, marginal areas are being reclaimed and continually degraded (Uganda 2006). Uganda’s human population has grown from 24.2 Million in 2002 (UBOS, 2002) to over 30 Million in 2012. At a 3.2% population growth rate (Nick, 2004), there is a great challenge of management of natural resources like wetlands. This poses a threat to *Eulophia*’s existence yet information on their ecology and genetics is still limited. Hence, the need to ascertain the genetic variation present which can be used to argue for conservation of the wetlands in future. The objective of the study was to determine relative levels of diversity among *Eulophia* in selected wetlands in Uganda measured using Amplified Fragment Length Polymorphisms (AFLPs).

### 2 Materials and Methods

#### 2.1 Study sites

Geographically, Uganda (in Africa) lies between latitude 4°N and 1°S and longitude 29.5°E and 35°E. Its average altitude is 1400m above sea level. About 1.3% of its total area lies above 2000m and 9.3% below 900m (National Environment Action Plan Secretariat, 1995; National Environment Management Authority, 1998). Africa’s wet areas are often rich in terrestrial orchids including many outstandingly beautiful tropical *Eulophia* species.

![Figure 1](image_url)

Figure 1 shows location of study sites in Uganda and the *Eulophia* species there in.
2.2 Sample size

The sample size was often limited by the actual sizes of natural populations as shown in table 1.

2.3 DNA extraction

DNA was isolated using the DNeasy Plant Mini Kit protocol. Dried young leaves from each individual were used. The dry plant material was ground in a 2ml microtube with stainless steel beads in a Tissue Lyser and ground for 2.5 minutes.

2.4 Restriction of the DNA

The AFLP procedure was performed essentially as described by Vos et al. (1995) 200 ng of genomic DNA was used. DNA was restricted for 2 hours at 37°C using 0.5µl EcoRI (5U), 0.5µl MseI (5U), and 5µl of 10X restriction – ligation (RL) buffer (100mM Tris HCl, 100mM MgAc, 500 mM KAc) in a final volume of 50µl.

2.5 Ligation of adapters

The adapter ligation was performed by adding the following to the restricted genomic DNA: 1.0µl EcoRI – adapter (5pMol), 1.0 µl MseI – adapter (50pMol), 1.2 µl ATP (10mM), 1.0µl10X restriction – ligation buffer, T4 DNA ligase (1U) and water to reach a final volume of 60 µl. The reaction was incubated at 37°C for 5 hours.

2.6 Pre-amplification

5µl of the digested and ligated DNA was mixed with 1.5 µl EcoRI primer + 1 selective nucleotide (75ng), MseI primer + 1 selective nucleotide (75ng), 1.0 µl dNTP mix (10mM), 5.0 µl 10X PCR buffer, 0.25µl Taq DNA polymerase (5U/µl) in a total volume of 50µl. The PCR reaction was performed for 30 cycles at 94°C – 30 seconds; annealing at 56°C – 60 seconds; extension at 72°C – 60 seconds and the run ended with 4°C for ever.

20 µl of the pre-amplification products were checked on 1% agarose gel. 100 bp ladder was used as the marker DNA. Ethidium Bromide was used during electrophoresis. Dilutions were made depending on the amounts detected on the gel. They were either 1:20 or 1:10 of pre-amplification: water. The diluted pre-amplification products were then used as template for the selective amplification.

2.7 Selective amplification

50 different primer combinations with three selective nucleotides were tested for the selective amplifications. 4 primer combinations were used on the samples. These were selected on consideration of the consistency of the results and the number of score-able bands. Adapters, MseI site primers and EcoRI site primers used are listed in table 2.

Final amplification was performed by using 0.5µl EcoRI primer + 3 (10ng/µl); 0.6µl MseI primer + 3 (50ng/µl); 0.4 µl dNTP mix (10mM); 2µl 10X PCR buffer; 2µl MgCl₂ (2.0 mM); 0.08 µl Taq polymerase to a final volume of 15µl of this mix. It was added to 5 µl diluted, preamplified DNA. The cycle profile was 1 cycle of 94°C – 30 seconds, 65°C – 30 seconds, 72°C - 60 seconds. The annealing temperature was then lowered by 0.7°C for each cycle for 11 cycles. This was followed by 24 cycles of 94°C – 30 seconds, 56°C – 30 sec and 72°C – 30 seconds. Followed by 72°C – 10minutes and ended with 4°C. The samples were amplified in a Perkin Elmer 9600 thermal cycler. The primers were selected on the basis of band amplification and reproducibility. In addition, primers showing optimum levels of polymorphism were used. Only strong and reproducible bands were scored and used in the final data analysis.

Table 1 showing the study sites, species and number of samples analyzed.
Table 2 Enzymes and primers used in the AFLP analysis.

<table>
<thead>
<tr>
<th>Adapter/primer</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>MseI adapter</td>
<td>5'-GACGATGAGTCTGTAG-3'</td>
</tr>
<tr>
<td></td>
<td>3'-TACCTAGGACTCAT-5'</td>
</tr>
<tr>
<td>EcoRI adapter</td>
<td>5'-CAGCCGCATTGACTACC-3'</td>
</tr>
<tr>
<td></td>
<td>3'-CAGGCATTGACTAC-5'</td>
</tr>
<tr>
<td>MseI + 1</td>
<td>5'-GATTGAGTCTGTAG-3'</td>
</tr>
<tr>
<td>EcoRI + 1</td>
<td>5'-GACGCATTGACTAC-3'</td>
</tr>
<tr>
<td>MseI + 3</td>
<td>5'-GATTGAGTCTGTAG-3'</td>
</tr>
<tr>
<td>EcoRI + 3</td>
<td>5'-GACGCATTGACTAC-3'</td>
</tr>
</tbody>
</table>

2.8 Silver Staining

Silver staining was done using a slightly modified protocol described by Bassam et al. 1991.

2.9 Genetic diversity and distance analysis

For an efficient genetic diversity, and distance analysis, prescreening of a few individuals was done. Using the prescreening, the most informative fingerprints were selected i.e. the fingerprints that would differentiate between the highest number of individuals. Gels were scored for the presence (1) or absence (0) of bands. Only those bands that could be scored confidently on all gels of one primer combination were recorded. After generation of the marker data set, pairwise comparisons of individuals by means of Jaccard coefficients (Jaccard, 1908) was done. Principal coordinates analyses (PCO; Gower, 1966) and cluster analyses (unweighted pair-group method using arithmetic averages [UPGMA]; Sneath & Sokal, 1973) were carried out. NTSYSpc 2.01 software was used (Rohlf, 1998). The dendrogram was generated to clarify diversity and genetic relationship.

An additional measure for partitioning genetic variation was obtained by Analysis of Variance (ANOVA).

3 Results and Discussion

Genetic variation is necessary for the evolution of species in their environment (Frankham 2005). However, natural populations of the majority of wild plant species show little divergence. The AFLPs used in this study showed diversity among individuals. Different primer combinations revealed independent subsets of this variation although sample sizes of *Eulophia* were limited.

The results show that *Eulophia* species are clearly rich in diversity at the DNA level. Figure II shows that *E. guineensis* of Kasozi are distantly related genetically to the others. Similarly, *E. horsfallii* of Mulehe and Mutanda are more similar to each other than the others of the same species in other areas. *E. horsfallii* of Pride and Kajansi show fewer variations among them compared to those of Mutanda and Mulehe. *E. angolensis* of Degeya, though in the same group with the others of same species in Njagalakasayi and Myanzi, it stands out clearly as genetically different, great variation is also reported in this species in Myanzi. *E. angolensis* of Nankalwe also stands out as distantly related to the others of the same species. *E. subalata* of Degeya is also clearly separated from the other species. The groupings are highly congruent with their geographical locations as individuals from same area were placed in one cluster and the different species separated from each other.

Each of the primer combinations generated fingerprint patterns markedly distinct from the other primer combinations. The combined data set used was based on 299 markers and 102 Operational Taxonomic Units (OTUs). Cluster analysis of all the AFLP data using 4 primer combinations with UPGMA based similarity coefficient is presented in a dendrogram that very closely conformed to the different populations (figure II). Populations were mainly not homogeneous for measured genetic diversity, although as reported in Travis et al., (1996) variance in population size may also contribute to the heterogeneity among populations. Comparisons of AFLP patterns revealed greater similarity among *Eulophias* collected from the same site.

The genetic diversity among individuals of different microhabitats reflect the tolerance ranges of each population beyond the range of any individual as also reported by Huenneke, 1991. Similarly, Eckert (2002) discussed that the cause of variation may be the type of reproduction and the possibility of variation is higher in the perennial plants that reproduce by sexual reproduction. In this regard, the low genetic variation in *Eulophia* populations can be justified if asexual reproduction is the common mode of reproduction.
This especially applies to *E. guineensis* at Kasozi. It is possible that sexual reproduction has mainly occurred in the past whilst vegetative reproduction via pseudobulbs has become increasingly dominant within their isolated habitat. The small population sizes and scattered distribution indicate that gene flow between populations is infrequent or even absent altogether as also reported in Milligan et al. (1994) in reference to loss of genetic diversity.

For rare or endangered species, a small sample size is unavoidable. This could lead to a significant bias in the population genetic estimates (Fischer et al., 2003). However, simulation results show that a sample size of 10–15 individuals is adequate for largely unbiased results (Isabel et al., 1999). In the study reported here, this sample size range applied to only 38% of the sites (Table I).

Individuals within populations sharing a multi-locus genotype are assumed to be asexually produced members of the same clone. Lowest genetic diversity was noted within *E. guineensis*. Probably due to the small numbers of individuals sampled, there may be an under representation of rare alleles as reported in Travis et al., 1996 for *Astragalus cremnophylax* var *cremnophylax*, a critically endangered plant. However, geographic isolation and small size may have led to loss of alleles and lower heterozygosity through genetic drift and inbreeding. Although it may have the advantage of maintaining its genetic characters, there could be drastic reduction in variability of this species. This would mean that long-term population persistence for this species may be hampered.

The variation noted among sites with similar species seems to be a function of the distance between collection sites. Genetic diversity was distributed within sites probably suggesting gene flow between populations within a given range. In this regard, the highly varied *E. harsfallii* of Mutanda and Mulehe, *E. latilabris* of Namwape and Kawanda, and *E. angolensis* of Myanzi show higher levels of adaptability and may not be easily passed out in case of environmental changes due to the expected increased gene flow between populations. Rare long
distance dispersal events determine both the possibilities of colonization of new sites and the structure of populations. They may also contribute to gene flow among populations as reported in Avise, 1994 and thus influence the distribution of genetic variation.

All individuals showed some level of genetic diversity except for the two pairs of identical plants recorded in the Sezibwa *E. latilabris* and Kasozi *E. guineensis* collections. These two sites had least variations between its members as shown in figure II.

Individuals that shared the same phenotypes were only found within populations and not between populations.

3.1 Analysis of Variance

To complement the information obtained from the clustering analysis, analysis of variance was carried out on the coefficients of the similarity matrices calculated for each population (site). This is given in table 3.
Table 3 Analysis of Variance within and between *Eulophia* samples.

<table>
<thead>
<tr>
<th></th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>4891.709</td>
<td>27</td>
<td>181.174</td>
<td>5.614</td>
</tr>
<tr>
<td>Within Groups</td>
<td>23622.286</td>
<td>732</td>
<td>32.271</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>28513.995</td>
<td>759</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The above table shows that the variance values between and within the samples are sufficiently dissimilar. There is therefore a significant difference between the samples at the 5% level. Genetic diversity was evident among and within populations. The significant between population variance probably indicates restricted gene flow.

The PCOs three dimensions account for 13.5%, 10.2% and 8.1% of the observed variation respectively. In the ordination analysis, nine groups were delimited as shown in figure III. In the PCO plot, the species found at Kasozi, *E. guineensis* and at Sezibwa, *E. latilabris* were most genetically separated from all the others. Sites with same species were closely positioned. It conforms to the separations of sites and species as in figure II. Generally, geographically closely located subpopulations were clustered together. It is therefore likely that propagation through rhizome fragmentation, common among the *Eulophia* appears to provide limited or little opportunity for long distance dispersal of genotypes.

High genetic differentiation existing among the populations studied suggests that population structure of the species is probably determined by colonization dynamics. The study revealed a level of genetic variation within and among sites. Isolation by distance of populations of plants of the same species may also have contributed to the variation observed.

**Conclusions**

Using the AFLP method, it was possible to ascertain that there is a wide genetic variation between and within the selected species of the genus *Eulophia*. Much of the *Eulophia* species genetic variation resides both within the sites and among populations. Such factors as population size and colonizing ability of the species, often similarly affect the amount of within population variation and population differentiation.

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