RECONSTRUCTION OF PHYLOGENY OF BIRDS OF THE GROUP ESTRILDIDAE BY USING DNA COMPARISONS AND DNA MARKERS

THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSPHY IN ZOOLOGY

by

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DEPARTMENT OF ZOOLOGY

ST.JOSEPH'S COLLEGE, DEVAGIRI

KOZHIKODE, KERALA – 673 008

OCTOBER – 2004

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CERTIFICATE

This is to certify that the thesis entitled **"The Reconstruction of Phylogeny in birds of the group *Estrildidae* by DNA comparisons and DNA markers"** submitted to the University of Calicut by **Smt. Thachappully Asokan Ligi** in partial fulfillment for the award of the degree of Doctor of Philosophy in Zoology is a bonafide record of the research work carried out by her under my supervision and guidance and that neither this thesis nor any part of it has formed the basis for the award of any degree or diploma.

Dr. V.J. Zacharias
Supervising Teacher

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OCTOBER – 2004

October 2004

DECLARATION

I, Thachappully Asokan Ligi do hereby declare that the thesis entitled " **Reconstruction of Phylogeny of birds of the group *Estrildidae* by using DNA comparisons and DNA markers"** submitted by me for the award of Doctor of Philosophy in Zoology of the University of Calicut is an original research work done by me under the supervision of Dr. V.J. Zacharias in the Department of Zoology, St. Joseph's College, Devagiri, Kozhikode; and that this work has not been submitted earlier in part or in full for award of any degree or diploma in this or any other University.

Thachappully Asokan Ligi

Candidate

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ABBREVIATIONS

µg	Micro gram.
µl	Micro litre.
AFLP	Amplified Fragment Length Polymorphism.
APS	Ammonium persulphate.
bp	Base pair.
cm	Centimeter.
DAF	DNA Amplification Fingerprinting.
DNA	Deoxyribonucleic acid.
dNTP	deoxynucleotide triphosphate.
EDTA	Ethylene Diamine Tetra Acetic acid.
M	Molar.
mg	milligram.
MHC	Major Histocompatibility loci.
ng	Nannogram.
PCR	Polymerase Chain Reaction.
RE	Restriction Enzymes.
RFLP	Restriction Fragment Length Polymorphism.
RNA	Ribonucleic acid.
SDS	Sodium dodecyl sulphate.
SNP	Single Nucleotide Polymorphism.
SSCP	Single Strand Conformation Polymorphism.
TBE	Tris – Borate EDTA.
u	Units.
UPGMA	Unweighted Pair Group Method using Arithmetic averages.
USGS	United States Geological Survey.
UV	Ultra violet.
VNTR	Variable Number Tandem Repeats.

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CHAPTER - I

INTRODUCTION

1.1 PHYLOGENETIC CLASSIFICATION

1.2 CLASSIFICATION OF ESTRILDIDAE

1.3 CONVENTIONAL TAXONOMY

1.4 DNA MARKERS

1.5 AIM OF THE STUDY

RECONSTRUCTION OF PHYLOGENY OF BIRDS OF THE GROUP *ESTRILDIDAE* BY USING DNA COMPARISONS AND DNA MARKERS

1. INTRODUCTION

The Reconstruction of Phylogeny by the construction of evolutionary trees is a key technique in answering a diversity of interesting questions especially in the field of management and conservation of wild life. Besides providing a basis for classifying living groups, it also helps to trace the route of transmission of a disease in epidemic; it can decipher the recent events in living populations, it is important for assessing prospective evolutionary potentials as well as for risk assessments and conservation strategies and also helps to establish the evolutionary relationships among all organisms.

1.1: PHYLOGENETIC CLASSIFICATION.

Organisms today are the products of evolutionary history that included the transformation, splitting and divergence of lineages, which resulted in the speciation. The evidence of evolution will therefore be preserved in some form in their living descendents. These evidences should be interpreted correctly. Morphology is an obvious source of evidence of relationship because we can expect closely related organisms to be similar in structure. But morphological characters are prone to convergent evolution, i.e. unrelated organisms come to look alike because their bodies have become adapted to cope with the same environmental demands through natural selection. Therefore convergence is a hazard in all comparisons, whether in living or fossil organisms. The fossil records are also not satisfying since

fossil record for most groups is fragmentary; and for some nearly non-existent. Partial phylogenies for some groups have been reconstructed from fossils, and it is possible to date most fossils with reasonable accuracy. However, a dated fossil records the time the individual organism died, not the time its lineage diverged from that of its next nearest relatives.

According to population genetists, genetic structures of populations show considerable genetic diversity that became apparent in the electrophoresis studies of proteins and DNA sequence analysis. The geographic ranges of most species also encompass a degree of environmental diversity. As a result of adaptations to different environments and genetic drift, within a species different populations will have different alleles or allelic frequencies at many loci. At this point it is the gene flow among populations that tend to homogenize their genetic composition.

But when the gene flow is reduced genetic drift and adaptation to different environments can cause populations to diverge. Eventually, population may become so different that the individuals in one population either will not or cannot mate with the individuals in the other. At this point the divergent population will become a different species. Thus speciation is associated with genetic divergence. Therefore it is possible to use the genetic differences among species to infer their evolutionary history. By comparing the amino acid or nucleotide molecular sequences it is possible to determine the genetic distances among species. These genetic distances are used to construct the evolutionary trees. The simplest methods for reconstructing phylogenies are based on the assumption that the least divergent species are one another's closest relatives.

Most recent studies on arrangements of the higher categories – Beta Taxonomy (Mayr et al., 1953) are now aimed at reconstructing evolutionary history – Phylogeny. But phylogenic studies are particularly

challenging in that they require a synthetic approach and a critical judgment. Evolution through natural selection has a very important corollary of convergence. Similar selection pressures have frequently acted on very different phyletic lines to produce superficially similar groups. This tends to obscure true relationships. Hence a systematist's most difficult job is to determine which characters have the greatest phylogenetic significance.

The classification of over 9000 bird species has been primarily based on morphological and anatomical features and had worked well. But since the last couple of decades DNA hybridization and other molecular techniques have refined avian systematics. These new techniques have caused us to look into the genetic and evolutionary relationships of birds as the primary tool for classification and nomenclature (Sibley. C. G., 1994) Though it has validated previous ornithological classification schemes to a large scale, presently the classification of birds is in a state of turmoil following the publication of 'Distribution and taxonomy of birds in the world' by C.G. Sibly and B.L. Munro in 1988 based on DNA affiliations. In spite of this, no attempt has yet been made in the subcontinent to revise bird taxonomy in the molecular line. The proposed project has been taken up as a pioneer project in the light of this current status of bird classification and nomenclature in India.

The avian fossil record is less complete than it is for other groups of vertebrates because bird bones are fragile and most birds tend to die in circumstances not conducive to fossilization. As relevant fossil evidence is lacking and the morphological and anatomical comparisons reveal little or produce conflicting opinions clearly a method that is independent of fossils and morphology, not prone to convergence, and that can measure the genealogical distances between living species should alone improve the reconstruction of avian phylogeny. Since genetic variation is the raw material

for evolution to measure the degrees of genetic variation analysis of the genetic material alone is the method.

Advances in molecular techniques, particularly the development of DNA sequence determination, have led to an explosive increase in studies of avian phylogeny. Such methods are independent of fossils and morphology and are not prone to convergence. Samples of blood, tissue, feather and even dried skin can be used as a source of DNA (Kajita, M. 1991) DNA sequences are then analyzed in the order of extraction, amplification, and sequence determination. Inferences about phylogenetic relationships from sequence data are made using an assortment of methods that include parsimony, maximum likelihood and distance. The confidence levels of the resulting phylogenic trees are then determined using statistical methods. Though much progress has been made in this line in countries like United States of America and Japan, practically no work has been carried out on similar lines in India.

There are many unsolved problems and many fruitful areas for study in the phylogeny of Indian birds. DNA analysis is a requisite method to solve these problems and phylogenetic analysis using DNA sequencing will play a leading role in solving questions relating to avian evolution.

1.2: CLASSIFICATION OF ESTRILDIDAE.

Munias of the family Estrildidae, also known as the waxbills; are common cage birds in India and abroad. As a result they are subjected to much admiration. Commonly called the waxbills or the weaver finches, most of them from Southeastern Asia and New Guinea belong to the genus Lonchura. They have strong black or bluish gray beaks. The plumage is mostly brown, black and white and in many cases with yellow or reddish brown edges on the tail feathers. The central tail feathers are pointed in most

species and hence the name Lancet tails and the scientific name Lonchura of the genus.

In addition to many substantive names, many use 'finch', 'sparrow' and 'weaver'; the members of the family are wide spread in the warmer parts of the old world from Africa to the Philippines, New Guinea, Australia and the Pacific Islands east to Fiji and Samoa. According to Delacour (1943) there are 125 species in 35 genera and many are well known cage birds.

The taxonomic arrangement of this group of birds was established by Delacour in 1943, who subdivided them into three tribes, the Estrildae or waxbills chiefly distributed in Africa; the Erythrurae or grass finches, occurring mainly in Australia and the Amadinae or the mannikins, occurring predominantly in South Asia. The weaver finches, or the Estrilid weavers are now grouped under the family Estrildidae (Passeriformes, suborder Oscines) that includes the waxbills, grass finches and mannikins (Delacour, 1943). This basic arrangement was followed by Mayer et al (1968), who recognized the Estrildae, the Poephilae, and the Lonchurae. The number of species, considered as comprising Estrildidae has remained rather stable (between 127 and 132), however the number of genera, largely differs between authors, 15 (Delacour 1943), 27 (Mayer et al, 1968, Goodwin 1982), to 48 (Wolters 1975-82).

The latest revision by Goodwin 1982 recognizes 137 species and 27 genera. The family consists of 3 main groups, the waxbills, the grass finches and the mannikins. Among the Waxbills there is considerable adaptive radiation of length 90 – 35 mm with 60 species placed in 16-17 genera of which some genera are poorly differentiated (Hall and Moreau, 1970). The Grass finches (100-130mm) are a rather heterogeneous group and include the majority of Australian members of the family with 15 members in 7 genera. Finally the mannikins in general are heavier looking than the others

(95-120mm), somber colored mainly brown or nearly black with bars or shaft streaks whereas some have large bold areas of black white and brown.

The taxonomy of these birds however cannot be discussed without reference to their avicultural history. Although the avian taxonomy has developed based chiefly on morphological and zoogeographic data, the classification of the Estrildidae relies much on additional knowledge obtained from caged birds. These have been for example behavioral observations (Morris 1954, Moyniha & Hall 1954, Goodwin 1960, Immelmann. K., 1965), studies of the mouth markings of the chicks (Mitchell,1958., Steiner,1960., Kunkel & Kunkel 1975) and research on vocalization (Hall 1962, Harrison 1962,Zann 1975).

In 1985, R.Kakizawa and M.Watada had analyzed 42 species belonging to 17 genera by means of protein electrophoresis of 26 loci to determine the evolutionary genetics of Estrildidae.With the genetic variation and differentiation obtained they were able to divide Estrildidae into two main groups, the Estrildinae and the Lonchurinae. The Estrildinae is comprised of three groups, the Estrildi, the Eruthruri and Amadini, while the Lonchurinae consists of the Poephili, Lonchuri, and Heteromunii. Though the basic taxonomic arrangement of genera and species agreed with current classification, the experiment had proved that Amadina and Lonchura to be an old separately differentiated group. Within each group the average inter specific genetic distance results supported the hypothetical African origin of the Estrildidae with later dispersal to Asia and Australia.

1.3: CONVENTIONAL CLASSIFICATION.

The conventional procedures and practices in systematics are reviewed best by Simpson (1961) and Mayr (1969a) with the modern theory of speciation. The detrimental effects of confusing the functions and goals of systematics and taxonomy are abundantly evident in the literature of avian

systematics, notably in that dealing with subspecies and hybridization. This duality is the prime basis for the current conflict between proponents of the 'phenetic' and 'phylogenetic' approaches to taxonomy (Colless, 1969). Recent developments in evolutionary theory and newer methods and procedures in systematics has brought out controversial aspects of taxonomic philosophy (Mayr, 1965) And these relate to problems at the levels of the subspecies, species and genus where there are obvious disparities between definition and application of concepts.

Family Estrildidae formerly called as the weaver finches were often grouped among the weavers in the family Ploceidae since their outer most primary, which is reduced as the true weavers, and the egg white protein patterns of the two groups are similar (Sibly, 1970). But many studies have revealed marked differences between weavers and weaver finches and hence grouping them in two different families has been widely accepted.

The waxbills were originally considered to be closely related to the families of the weavers (Ploceidae) and weaver finches (Estrildidae). So they were formerly classified as a subfamily of the Ploceidae (Chapin 1917, Sushkin 1924, 1927). Although he also considered the waxbills to be a subfamily of the Ploceidae, Delacour (1943) emphasized a distant link between the Ploceinae and the Viduinae. Subsequently, the waxbills have been treated as a separate family (Beecher 1953, Wolters 1957).

Conventionally, some of the African and all of the New Guinea species are referred to as Mannikins and most of the Asians as Munias (Restall 1996a). Delacour (1943) gives a comprehensive review of the divisions of the group. He considered there to be three super genera; Padda, Amadina, and Lonchura. Delacour's (1943) work was the basis of all subsequent revisions for the next few decades. Subsequently Wolters (1957) Steiner (1960) and Guttinger (1976) gave further conclusions. The nomenclature and sequence used by Peters Checklist of the birds of the world (Mayr & Paynter 1968) is widely used today. Mayr and Paynter (1968)

recognizes two genera, Padda and Lonchura and Goodwin(1982) merges Padda with Lonchura. Sibley and Monroe(1990) recognize Heteromunia (Pictorella Mannikin) Lemuresthes (Madagascar Mannikin) and Padda in addition to Lonchura. Restall (1996b) used the name Lonchura for the genus throughout.

There is not much agreement on the generic limits and detailed taxonomic arrangement of the weaver finches, partly because of the great diversity of plumage pattern, sometimes even between birds very closely related in behavior. Three tribes - the waxbills (Estrildini), grass finches (Erythrurini) and mannikins (Amandini) – are however recognized by Delacour (1943) and variously combined by others.

The waxbills are almost entirely an African group except for the two species of avadavat in India and the Orient and one species of problematical affinities, the Sydney Waxbill *Estrilda temporalis*, in eastern Australia. There is a considerable adaptive radiation in the African forms, there being many small seed –eaters, large seed eaters, arboreal insect eaters and the little flower pecker finch *Parmoptila woodhousei*, formerly put with the flower peckers Dicaeidae. (Chapin,1917) Only few of the small waxbills of the genus Estrilda and some relatives have been studied in captivity, and the diagnostic behavioral characters of this tribe given by Delacour seem to refer only to them. From the summary of his observations on waxbills the palate markings are spots reduced in a few; the courtship includes carrying a straw in the mouth and there are usually reflective nodules on the gape. Most of them are rather brilliantly colored.

Grass finches include the well-known cage birds, such as the Gouldian finch *Poephila gouldiae*, Zebra finch *P.castanotis*, Parson finch *P.cincta*, and Diamond sparrow *Zonaginthus guttatus*. The character that holds this group together is that they are predominantly Australasian, and certainly Estrilids but not manikins in Delacour's view. The characters given to the tribe amount to no more than their geographical distribution, harsher

voices than in Estrilda, and great variation of palate markings from a typical domino pattern as in Estrilda through combinations of spots and horse shoes to as good a pair of horseshoes as is shown by any manikin. In plumage they are extremely diverse, ranging from the brilliant crimson, black, white, and brown of the painted finch *Zonaginthus pictus*, or the iridescent green unique in Estrildids and reds or blues of Gouldian finch and the parrot finches; Erythura spp., to the dull browns and the pale grays and purple of the cherry finch *Poephila modesta.* In consequence, in some revisions, nearly every species is put into a separate genus or sub genus. It is quite possible that most are really waxbills but that a few are modified mannikins.

Mannikins are a much more homogenous group. As a rule they are rather heavy looking birds as compared with Estrilda. (Wendt and Wolters, 1973). Most are quietly coloured in browns and nearly black with bars or shaft streaks and some have large bold areas of black, white, and brown (the tri color nun. *Lonchura malacca*). In the Cut- throat *Amadina fasciata* and Red headed Finch *A. erythrocephala* there is a brilliant red on the head. The largest is the well-known Java Sparrow *Padda oryzivora.* Most are found in the hotter parts of the Orient, the Malay Archipelago , New Guinea, and Northern Australia, but a few extend to India and Ceylon and there are three groups in Africa. These are the Magpie mannikin *Lonchua fringilloids*, the Black breasted mannikin *L. bicolor*, and the Bronze wing mannikin *L. cucullata*; and the two silverbills, of which the common silverbill *Lonchura malabarica* extends to Arabia, Persia, and on to Ceylon. There is, lastly, the curious Bib finch *Lonchura nana* in Madagascar.

So far as is known, the palate markings are two horseshoes in all but Amadina, which has five large blotches with only narrow ridges of white between them. The silverbills are rather exceptional in their high pitched voice. Several of the Oriental forms such as the Spice finch *Lonchura punctulata* and Striated finch *L. striata*, are very common birds, sometimes pests of grain crops. The ' Bengalese finch' is a very well known cage bird,

excellent as a foster parent for other seed eaters, and known only in captivity; Eisner (1957) has shown that it is a cultivated form of a sub species of the Striated finch.

In the natural condition, munias are birds of arid Savannah and open grassland. Fresh adult plumage is often brightly colored. According to Goodwin (1982) bills of estrilids tend to increase in size disproportionally as the birds get larger.

Since Goodwin (1982) a great deal of work has continued, mostly in Germany, studying and breeding individual species. The study of Kakizawa and Watada (1985) at the Yamashina Institute in Tokyo is particularly interesting. The authors analyzed the genetic variations of 42 species of estrilids by means of protein electrophoresis.

In India no work has been carried out on the phylogenetic classification of this group of birds. Most of the works on the birds of the family were carried out on their role as agricultural pests. (Ali, et al 1976, Shivanarayanan, N. 1984, and Bargava, R. 1999).

1.4: DNA MARKERS.

Genetic variation is the raw material for evolutionary change. The sum of the genetic variation present in a population or species is referred to as the gene pool. A variety of techniques ranging from simple visual inspection to protein gel electrophoresis, RFLP analysis, RAPD, and DNA sequencing may be used to estimate the level of variability in a population or species. (Klug and Cummings, 2003). The amount of genetic variation in a population may be expressed as the percentage of polymorphic loci or average heterozygosity (H). Extensive surveys conducted in a great number of species over the past several decades indicate that most populations harbor substantial levels of genetic variability. This built in genetic diversity is concealed and not apparent in the phenotype. Hence, detecting them has not been a simple

task. Nevertheless with the help of techniques mentioned below such investigations has been successful.

Heritable traits that can be assayed (markers) are of many different kinds:

- **Visible :** (physical appearance): height, shape, color, growth rate, etc.
- **Auxotrophic:** (growth requirements). For microorganisms such as baker's yeast, though some markers are visual markers affecting colony color or morphology, many are auxotrophic markers
- **Antibiotic:** sensitivities and resistances.
- **Conditional**: nutrition (auxotrophy); temperature (ts mutants); light; developmental; etc.
- **Molecular :** Characteristics of several biomolecules can serve as markers on the genetic chromosome
- **Antigenic**
  - Blood group antigens are carbohydrates.
  - Histocompatibllity antigens are proteins. Discovered via tissue transplantation, MHC loci are polymorphic.
- **Enzymatic**
  - Isoenzymes are proteins.
- **DNA-based**
  - RFLP's (restriction fragment length polymorphisms)
  - RAPD's (random amplified polymorphic DNA)
  - AFLP's (amplified fragment length polymorphisms)
  - Microsatellite repeats—VNTR's
  - SSCP's (single strand conformation polymorphism)
  - SNP's (single nucleotide polymorphism)

To develop management strategies for maintaining evolutionarily significant lineages that will ensure long-term population

stability and reduce the need for protection through the regulatory process, a thorough understanding of the evolutionary relationships (e.g., levels of gene exchange) among geographically proximate and distal populations are essential. Molecular genetics has recently achieved an important place in contemporary conservation biology as it has proven to be a robust tool for identifying reproductive isolation among populations, permitting the delineation of management units, and allowing assessment of conservation priorities from an evolutionary perspective. Co-dominantly inherited genetic markers are required to fully assess population structure, gene flow, kinship, introgression, hybridization, and speciation. USGS's Biological Resources Discipline conducts molecular systematics and population / metapopulation genetics research involving species of conservation concern.

Conservation biology seeks to maintain both unique species and the genetic diversity within those species. Without the help of molecular genetics, however, it's not necessarily easy to quantify either quality. Using mitochondrial DNA, Polymerase Chain Reaction techniques, and microsatellite DNA, molecular geneticists help biologists determine which species are genetically distinct and whether their DNA are adequately diverse, allowing wise management of endangered species.

1.5: AIM OF THE STUDY.

Ecosystem dynamics have identified systematics as the research priority that is fundamental to ecosystem management and biodiversity conservation. Therefore the primary need requires improvements and access to standardized nomenclature based on reliable information on species relationships and their hierarchal classification.

During the past 50 years, molecular biologists have developed techniques that make it possible to decipher the information encoded in the genes - the DNA of organisms (J.W. Lang et al. 1993) Evolutionary changes

at the genetic level involved changes in one or more letters of the genetic message. If a method for comparing the DNAs of different species can be developed it should be possible to assess the degree of genealogical relationship among living organisms. Much work has progressed on these lines in Europe, America, but practically no work has been carried out on similar lines on birds in India.

Differences between DNA reveal evolutionary distances between species making it possible to reconstruct and date the branching of avian linkages and providing a basis for classifying living groups. Reconstruction of phylogeny is the primary goal to evolutionary biology. To reconstruct the 'Tree' of life it is necessary to determine the branching pattern of life and if possible, the dates of branching events in the past.

Indian subcontinent harbors about 2000 species and sub species of birds. The Western Ghats is amongst the 18-biodiversity hotspots recognized globally. These hills are known for their high degree of endemism expressed at both higher and lower taxonomic levels. However, uncertainties that prevail in the taxonomy and nomenclature have frustrated biologists in exercises leading to conservation assessments in the Western Ghats (P. Pramod et al, 1997) Practically nothing is known about the taxonomic relations and phylogeny of Indian birds.

Conservation assessment and management programme have heavily relied on biodiversity and endemism as attributes. Several species of birds are endemic to the Indian subcontinent, while others have a wide distribution across west Asia, south East Asia and Africa.. Still a lot of controversies exist about the taxonomy and nomenclature of many of these birds. Reconstruction of phylogeny and establishment of taxonomic relationships and identity will remove the prevailing uncertainties in avian diversity and help in their conservation and management programme.

Knowledge about the identity through DNA study would be very much helpful in understanding the avian diversity, (Kajita 1999,Nagata

1999) which is necessary for the conservation and management of birds in India. Such studies on birds have been extensively carried out in countries of Europe N. America and Japan. (Parkes 1958, Christidis 1987, Restall 1996)But studies in this line have not been taken up on similar lines on birds in India.

As a first step in this field, a study based on the molecular methods in avian phylogeny is proposed on munias, which are represented by seven species in India. It includes two endemics - the Green munia and the Black-throated munia (Gaston,K.J. & Blackburn,T.M., 1997) The munias belong to the family Estrildidae (Ali S., 1996) They are common cage birds that do not interbreed in captivity. This study initiated with the group is expected to give a clear picture of the relationship and phylogeny of the seven different munia species found in India.(Manakadan and Pittie, 2001) Though isolated works have been carried out on some species of the family on account of their importance as chief agricultuaral pests of Indian fields (Verghese et al, 1981,Bhattacharjee et al, 1998), no studies have been reported on the phylogenetic classification of this family of birds in India. The method thus developed in the study will be used as a tool for studying other bird groups in India. It is expected to give a comprehensive picture of the taxonomy and phylogeny of these Indian birds.

In this study **RAPD** (Williams et al., 1990) was used for analyzing the genetic structure of the Munia populations and the genetic variability within and among them. RAPD stands for **R**andom **A**mplification of **P**olymorphic **D**NA. RAPD reactions are PCR reactions, but they amplify segments of DNA which are essentially unknown to the scientist (random). Polymorphic DNA are the DNA sequences which are relatively variable between different individuals or species. "poly" = "many" "morphic" = "shapes"

Some stretches of DNA within a genome will be extremely conserved (will not vary) among individuals. Such conserved DNA will not be

useful for analytical methods, which use DNA to find differences among individuals. Alternatively, some stretches of DNA within a genome tend to vary moderately or greatly among individuals. These variable stretches of DNA will be far more informative when using DNA to distinguish individuals from one-another.

RAPD was chosen because it is expeditious and inexpensive and does not require a previous knowledge of the genome of species under study. (Lynch and Milligan, 1994). Earlier comparisons showed that RAPD and isoenzyme results are very similar, with the RAPD technique revealing even higher amounts of variation (Baruffi et al., 1995).

RECONSTRUCTION OF PHYLOGENY OF BIRDS OF THE GROUP ESTRILDIDAE BY USING DNA COMPARISONS AND DNA MARKERS

THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSPHY IN ZOOLOGY

by

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2. REVIEW OF LITERATURE

Earlier classifications that were based on comparative anatomy had given a better understanding of relationships between groups. Now classifications are based on phylogenetic relationships between two groups and therefore classifications must be changed to keep up with the steady increase in our knowledge of the phylogenetic relationships among species and groups of species. (Storer, 1971).

2.1: AVIAN SYSTEMATICS AND TAXONOMY.

Although the terms systematics and taxonomy are often used interchangeably, there are important reasons for making a distinction by defining systematics as the study of the kinds and diversity of organisms and the relationships among them, and restricting taxonomy to the theory and practice of classifying organisms. (Simpson, 1961). The primary goal of systematics is the casual and historical analysis of diversity in terms of levels of integeration designated as natural populations (Mayr, 1968b), whereas taxonomy is concerned with schemes of classification reflecting this diversity. Conflicts arise from this duality of purpose, since the search for explanations of diversity often does not harmonize with the description, naming and classification of taxa (Sokal, 1964). Practical aspects of taxonomy are unimportant to evolutionary theory, and the use of speculative evolutionary interpretations of taxonomic data in establishing classifications may, according to Sokal 1964, ' remove taxonomy from the domain of the exact sciences.'

2.1.1.General Status.

Avian taxonomy was based largely on the morphological features of museum skins, but increasing attention is now being given to biochemical behavioral, ecological, physiological, and molecular genetic characters.

2.1.1.1: Morphological:

The most useful early classifications were those based on morphological similarities. For example, the importance of understanding the sequence and patterns of moults and plumages and rates of cranial ossification as bases for age determination is still widely recognized. (Pitelka, 1945; Selander and Giller,1960; Selander, 1964; Newton,1966; Scott,1967). Realizing that subjective evaluations of small differences in color are essentially meaningless, systematists have adopted methods of quantitative measurements. (Wyszecki and Stiles, 1967). For color comparisons, light provided by an illumination system employing fluorescent tubes is superior to skylight (Yang and selander,1968). f color must be specified in a more precise fashion than can be done by comparison of specimens with color chips or reference specimens , a spectrophotometer may be used to obtain diffused spectral reflectance curves from which color is specified in international units of brightness, dominant wave length, and purity (Selander et al , 1964;). With this technique, provisional identification of carotenoid pigments can be made from the position of peaks of reflectance (Dyke , 1966; Selander and Johnston,1967), and colors of feather pigments and soils can be compared (Johnston, 1966a).

Research on pterylosis and moult has provided little information of direct systematic value. Pterylosis is remarkably constant in the passerines, especially among the oscines (Ames et al, 1968), and extensive studies of moult by Stresemann and Stresemann (1966) have not furnished

evidence for judging phylogenetic relationships, even at the ordinary level (Johnson and Peters ,1963). Studies of Karyotypes of birds have not produced information of great systematic value. Ohno et al (1964) stressed the uniformity of the Carinatae (Bloom,1969). In a representative study , Castroviejo et al. (1969) found no differences in the sparrows *Passer domesticus* and *Passer montanus.* , but noted that the Titmice *Parus major* and *Parus palustris* differ in morphology of the Z chromosome and the fifth pair of autosomes. Minor karyotypic variation among species of the Turdidae and Corvidae has also been reported (Jovanovic and Atkins, 1969). Only a single case of chromosomal polymorphism in birds has been described (Thorneycroft, 1966).

2.1.1.2: Behavioral:

Behavioral characteristics were employed in the revision of the Anatidae by Delacour and Mayr (1945), and Johnsgard (1961a), Mckinney (1961) and others have extended this work , supplying comparative behavioral analysis of all genera and tribes. The adaptive value of morphological features , such as facial masks, camouflaged plumage, dark or light underparts, and of displays, vocalizations, and other behavior has been studied observationally and experimentally (Tinbergen et al.,1967). This work has demonstrated a complex interaction of selective factors influencing the evolution of displays; major evolutionary trends have been the reduction of interspecific and intraspecific ambiguity in signals and the adaptation of behavior to special environmental or ecological conditions. Behavioral characters are not invariably of great taxonomic value, even among the Anatids. Thus in linking Heteronetta with the Oxyurini (stiff tailed ruddy – ducks), Weller (1968) found that morphology was a better guide to relationships.

Because of the ease with which sounds can be recorded on tape and analysed spectrographically or in other ways, vocalizations are

extensively employed in systematic work (Mayr,1958; Lohrl,1963; Thielcke,1969; Lanyon,1969). Thielcke (1962) studied the vocalizations of the sibling species of tree – creepers, *Certhia brachydactyla* and *C. familiaris,* in Europe from the stand points of ontogeny and individual, geographic, and interspecific variation. In playback experiments individuals responded to vocalizations of their own species but usually not to those of the other species, or to those of the new world *C. Americana*. On this basis, Thielcke suggested that *C .Americana* is not conspecific with *C. familiaris*. In one experiment, *M. barbirostris* of Jamaica did not respond to the playback of certain vocalizations of its mainland relative *M. tuberculifer*; and, largely on this basis , the two forms were considered specifically distinct. Results of the play back experiments should be interpreted with caution. W.J.Smith (1970) stresses the importance of considering the biological relevance of vocalizations as part of adaptive social behavior. A basic limitation of playback is its failure to permit a normal behavior interaction to develop. Falls and Szijj (1959) found that the meadow larks *Sturnella magna* and *S.neglecta* learned to recognize each others songs presumably by associating them with agonistic interaction evoked by the similar plumages.

2.1.1.3: Biochemical:

Methods of studying the comparative anatomy of whole proteins or partial digests of proteins are being applied to systematic problems (Leone,1964; Feeney and Allison,1969; Dessauer,1969).Their contribution to avian systematics have been significant, especially as the genetic information in the amino acid sequences in the proteins become available.(Fitch and Margoliash,1969). The tools have become available for assessing systematic relationships among populations in terms of total numbers of allelic substitutions at genetic loci.

The ovalbumins and lyzozymes of a number of birds , mostly domesticated species, have been compared immunologically, using

microcomplement fixation and other techniques (Arnheim and Wilson,1967). The erythrocytic and serum antigens of pigeons, doves and several species of gallinaceous birds and ducks have been studied by M.R.Irwin and others (Irwin, 1964). Major attention has been given to the egg white proteins, partly because they are easily obtained and partly because of a widespread, although essentially unfounded belief that they are more conservative than other proteins. For example , immunoelectrophoresis , immunodiffusion and other biochemical techniques were used by Osuga and Feeney (1968) and H.T.Miller and Feeney (1964, 1966) to compare egg white proteins of the ratites and all evidences indicated that the Tinamours represented by *Eudromia elegans* are only remotely related to the other ratites.

Electrophoresis of proteins has not provided much information for avian systematics, particularly at the generic and specific levels. The investigations of Sibley (1960,1967) in this respect, on egg white proteins, using relatively crude technique of paper electrophoresis, were of greater value in indicating the potential usefulness of electrophoresis in systematics than in solving specific taxonomic problems. Starch-gel and acrylamide electrophoresis, providing superior resolution of protein bands , has replaced the paper technique . Using the starch-gel electrophoresis of egg-white proteins Sibley (1968) showed that the wren thrush Zeledonia, previously classified as a thrush and placed in a monotypic family, is a nine primaried oscine. And an interpretation of morphological data suggested that it is related to the warblers. In another study, Sibley et al (1969) concluded that all lines of systematic evidence , including starch gel electrophoresis of egg- white proteins and haemoglobin , indicate that the flamingos and Ciconiformes are more similar to one another than either is to the Anseriformes. The strongest evidence comes from thin-layer electrophoretic patterns of tryptic peptides of ovalbumins.

2.1.2: International Status:

Much work has progressed in the field of molecular taxonomy since the pioneering work in this field by Charles G. Sibley, Jon E. Ahlquist and coworkers in 1983 (Burke & Bruford, 1987). Their 1991 Life Science publication- Phylogeny and Classification of living birds - A study in molecular evolution, is a milestone in ornithology by virtue of its herculean scope and its pioneering methodology. It is the study of evolutionary history and classification of living birds based on comparisons of the genetic material of about 1700 species. Since then extensive works are being carried out in Europe, North America and Japan. India with its rich bird fauna that include several endemics has received much attention from ornithologists all over the world. (Stattersfield. A.J et al, 1998).

2.1.3:National Status:

Though the Indian subcontinent harbors about 2000 species and subspecies, practically nothing is known about their phylogeny and taxonomic relations (Wetmore. A, 1998) The classification of Indian birds have been undergoing periodic upheavals from the time the first edition of the ' Fauna of British India, Birds' was published in 1889-90 by Oates and Blanford. Later the publication of the Synopsis of the birds of India and Pakistan' by S. Dillon Ripley in 1961 and the subsequent publication of the 'Handbook of the Birds of India and Pakistan' by Salim Ali and Dillon Ripley between 1968-1974 based on the synopsis gave a certain amount of stability to the taxonomy of Indian birds. The latest centenary edition of 'The Book of Indian Birds' by Salim Ali describes 538 species of Indian birds. Still a lot of controversies exist about their taxonomy and nomenclature. Several species of birds are endemic to the Indian sub-continent while others have a wide distribution

across west Asia and Southeast Asia. So far molecular taxonomy based on DNA studies of birds have not been taken up in India.

Interest in the fate of endangered birds in the country has centered mainly on large, spectacular species such as the Great Indian Bustard *Choriotis nigriceps* and the Siberian crane *Grus leucogeranus*, or insular forms such as the Laysan Finch *Psittirostra cantans* or the various Hawaiian Honeycreepers. (Halliday, 1980). Small continental species do not generally merit such concern with a few prominent exceptions.

The resilience of continental passerine species with non-passerines stems from the high densities that they maintain, allowing adequate populations to survive in relatively small patches of habitat. However, with the continuing conversion of ecosystems from natural to manmade configuration, we may anticipate the fragmentation of species populations sufficient to qualify for the attention of the conservationists. Because of the antiguity of human settlements and cultivation in India and Pakistan and the very high density of population maintaining over many centuries over most of the area (Bose et al. 1965), natural ecosystems occurring in the Indo-gangetic plain and the Deccan plateau have been virtually eradicated. They have been replaced in areas suitable for agriculture with intensive cultivation and in most other areas with derelict scrub and semi desert communities (Eckholm,1979).

Based on the observations made over the twelve years throughout India and Pakistan, Gaston (1984) had examined the status of endemic passerines in the subcontinent and revealed that the species characteristic of the heavily disturbed low land ecosystems of the indo-gangetic plain and the peninsular India may show signs of increased rates of extinction, or vulnerability to extinction.

2.2: FAMILY ESTRILDIDAE.

They are mostly small to very small seed eaters and were grouped in Ploceidae in being Finch like and in having the outermost primary merely reduced. However they vary conspicuously from the weavers in most other characters and have been associated by different authors with other groups of finches, for various reasons, none of them very convincing. In view of their molecular and behavioral peculiarities, it seemed best to follow Steiner and consider them as a separate family.

2.2.1: Morphological:

Among the two genus considered , the Amandava (Estrilda) are brightly colored while the Lonchura are all somber brown, sometimes nearly black, with only barring and streaking. Sexual dimorphism is only sparsly evident except for the male breeding plumage noted in the Red munia. The juvenile plumage are mostly similar to that of the adult., but paler; or it may be very dull, mostly grey. Only the Red Munia, *Amandava amandava* has a non- breeding plumage in the young stage.

The nest is an untidy bundle, never woven like a weaver's; and it is firmly fixed among twigs, not suspended (Sykes, 1834). The entrance is short and horizontal. The eggs are 4-6 and pure white unlike in weavers, where 2-4 colored eggs are laid. The nestlings are remarkable for the decoration of their palates and tongues, which have conspicuous black spots, bands, or blotches often on a white or bright yellow background. The palate has often five black spots in a so- called domino pattern (waxbills and grass finches) or two black horseshoe marks (mannikins). The edge of the gape usually has conspicuous white or black and white swellings, sometimes with large nodules that can reflect the light and are presumably conspicuous inside

the dark cavity of the nest. The palate and the tongue markings are conspicuously displayed when the nestlings beg for food, which it does by a special crouching and waving of the head, the mouth being held wide open, very unlike the simple neck stretched gape of most passerines.

2.2.2: Behavioral:

Both parents take part in incubation, which lasts on the average 21 days. The nest, at least after the first week of nestling life, is not kept clean by the parents. The nestlings defecate on the walls of the nest, and the faeces rapidly dry and stick there, so that the nestlings are not fouled. Estrildids mature very quickly and may breed at the age of a few months, while Ploceids take much longer.

The Estrildids usually seem to form stable pairs, which share, in nest building, incubation and care of the young. They seem very often to be social, perching in clumps and indulging in much mutual preening not confined to individuals of the same species at least in aviaries (Taher,H.1995).

There is a considerable variety of courtship pattern in those Estrildids that have been studied, but in all there is an absence of wing quivering, common in other passerines; and a marked peculiarity is the use of tail – quivering as a female solicitation display. The courtship dance of the male may consist almost entirely of stiff-legged leaps upwards returning to the same spot each time , or rapid swings from side to side , or actions involving both these vertical and lateral motion. The ornamented areas of the plumage are usually erected and shown off. In many species the male may carry a long piece of grass or feather in his bill while dancing and most dances involve some action of the bill; like, clappering, jerky opening and closing of bill and some times tongue protrusion. The songs may consist of high pitched short phrases rather melodious and clearly audible in waxbills, toy trumpet notes in grass finches or un-melodius clucks, faint whistles, and slurs in a definite pattern as in the manikins, sometimes inaudible even a couple of feet away.

2.2.3.Biochemical:

In 1985 Kakizawa and Watada had carried out protein electrophoretic studies of 26 loci to determine the genetic variation and differention of 42 species of Esrildidae and constructed a genealogical dendrogram by means of the UPGMA procedure. Based on the values of interspecific distances the dendrogram revealed that the family Estrildidae is divided into two distinct groups. One group fits well with the currently recognized waxbills (Estrildinae), and the other comprises the so called mannikins (Lonchurae) , and grassfinches (Poephilae). They recognized the two groups as subfamilies Estrildinae and Lonchurinae each consisting of three subgroups.

Estrildinae consisted of the cut-throat and red-headed finches, the Waxbills (the Estrildae with other waxbill genera) and the parrot finches (Erythrura and Chloebia). The Lonchurinae consisted of the species Heteromunia pectoralis, the Grass finches (Poephilae of Mayer et al, 1968) and the Mannikins(Lonchura and Padda). In their classification, Erthrura, Chloebia and Amadina which belonged to Lonchurae in Mayer et al (1968), are moved into the Estrildinae. But no species have been transferred from the Estrildae as recognized by Mayer et al, (1968) to the Lonchurinae.

2.3: GEOGRAPHICAL DISTRIBUTION OF ESTRILDIDAE.

The true Waxbills are confined to the tropical region where they are mostly found in open savannas and clearings within the forest areas. The common waxbill of the genus Estrilda; *Estrilda estrilid* is widespread in the south of about 5 degrees North in E. Africa and has been introduced to many oceanic islands, South American mainland and Portugal. In W. Africa the Black rumped waxbill *E. troglodytes* take its place. *E. melpoda* is another attractive bird of forest clearings and swampy areas of West and Central

Africa. Whereas, the south and east of its range is taken by Fawn breasted waxbill *E. paludicola*. Genus Lagonosticta include seven species of fire finches distributed in Sengar, Sudan and Ethiopia to Natal and along the warm valley of the Orange River almost to its mouth. The Red-bellied fire finches *L. senegales* is found frequently entering houses in towns and villages. Genus Pytilia has five species of striking birds found in thorny thickets and undergrowth in open savanna . Of this the Melba finch *P. melba* is the most beautiful.

Birds of the genus Cryptospiza are found in the montane forests of the east and central southern Africa. The Negro finches of genus Nigrita and flower peckers of genus Parmoptila are found in primary and secondary lowland forests of Africa.

The waxbills are almost entirely an African group except for the two species of avadavat in India and the Orient and one form of avadavat of the Asian waxbills, the green avadavat, *Amandava formosa* (*Estrilda formosa*) an endemic of India is unique in being olive green above and yellow underneath from chin to tail. It's home is central India. In contrast, the male red avadavat *Amandava amandava* (*Estrilda amandava*) in breeding time is crimson below spotted with white, red or brown above and has brown wings. It is the only Estrilid that moults into a duller non-breeding plumage. It occurs throughout India, West Pakistan and southern Nepal, and its range extends eastwards to Cambodia, Saigon, and the Lesser Sunda Islands (Biswas et al.1963). It has been introduced into numerous islands such as Mauritius , Fiji, Sumatra, Singapore and the Philippines.

The grass finches are predominantly found in Australia. Most of them are birds of open grasslands and savannas, usually in the vicinity of water. Four species however occur in specialized habitats. The Blue faced parrot finch *Erythrura trichroa* at the forest edge in northern Queensland, Red eared fire tail *Emblema oculata* in dense eucalyptus forests in south west Australia; and the Painted finch *Emblema picta* and Zebra finch *Poephila*

guttata in semi deserts in central Australia. In plumage they are extremely diverse. The Parrot finches (12 species) are wide spread in the Philippines, Borneo, New Guinea and various islands in Melanesia, some of which have endemic species.

The Mannikins are somber colored, mainly brown or nearly black and white in many cases with yellow or reddish brown edges on the tail feathers. Most of the Mannikins of the southeastern Asia and New Guinea belong to the genus Lonchura. There are 28 species ranging from India to Australia. Five of them are widely found in India and commonly called as the Munias. (Salim Ali, 1968).

Munias are birds of the family Estrildidae, having the size of sparrows; the largest being the Java sparrow and the smallest, the Madagascar mannikin. According to Restall (1997b) they are widespread throughout the Afro tropical, Oriental, Australasian and Melanesian regions. They range from West Africa to the Arabian Peninsula and through Asia to China, Southward to New Guinea and Australia and even beyond, to some of the Pacific islands, where one species is found on the island of Pomade in the Carolinas. However some of them are found almost all over the world; introduced by accident or by design.

There are 28 species ranging from India to Australia. Many are well known domestic cage birds in Europe. Among them are the Spice Finch *Lonchura punctulata*, which occurs from India to Taiwan and the lesser Sunder Island. The sharp tailed munia *Lonchura striata* is believed to be the ancestral form of the domesticated society finch. Several centuries ago the Chinese had bred the society finch, which became a completely domesticated bird called the society finch from the sharp tailed munia. For about 250 years breeding of the society finch was carried out mainly in Japan and for the past hundred years also in Europe. Ernest Schafer describes the behavior of the southern Chinese subspecies of the sharp tailed munia *Lonchura striata swinhoer* as extraordinarily sociable bird who like to keep to the edges of rice

fields, weedy gardens, pastures, cemeteries and near temples quite fearless often letting people to approach near them. The three-colored mannikin , *Lonchura malacca* is found from India and Ceylon to the Philippines, Java and Bali. The White Throated munia *Lonchura malabarica* is a common munia seen in south India. *Lonchura kelaarti* or Black throated munia is the endemic species found restricted to the high ranges of the Western Ghats.

Lonchura punctulata (spice finch or spotted munia) occurs from India to Taiwan and the Lesser Sunda Islands. The sharp tailed munia or the white backed munia, *Lonchura striata* occurs from India and Ceylon to Southern China and Sumatra. *L.malacca,* black headed munia or three colored mannikin is found from India and Ceylon to the Philippines, Hal mahera, Java and Bali. *L.malabarica,* the Indian silver bill, white-throated munia occur in India and Sri Lanka. Where as *L.kelaarti,* Rufousbellied munia or the hill munia; (Pande. S., et al., 2003.) an endemic species is found restricted to the high ranges of the Western Ghats and Ceylon.

2.4: SPECIES OF ESTRILDIDAE FOUND IN THE SUB CONTINENT.

In the Indian subcontinent seven species are widely described (Ali.S.1996). They are commonly referred to as the munias. But of the seven species two belong to genus Estrilda and the remaining five are grouped in genus Lonchura. Munias of the subcontinent as listed by Ranjit and Pittie, 2001 are *Amandava amandava (Linnaeus,1758), Amandava formosa(Lathan,1790), Lonchura malacca(Linnaeus,1766), Lonchura striata(Linnaeus,1766) Lonchura punctulata(Linnaeus,1758) Lonchura malabarica (Linnaeus,1758)*, and *Lonchura kelaarti (Jerdon,1863)*,. Of the seven munias *Amandava formosa* and *Lonchura kelaarti* are found to be endemic in India. Genus Amandava (Estrilda) include the true waxbills, and are mostly colorful whereas Genus Lonchura includes the mannikins that are somber coloured.

The two common cage munias of India belong to the genus Estrildae, or Amandava and are commonly called the Avadavats (Sharme et al 1987). They are far hardier cage birds. The green avadavat (*Amandava formosa*) is olive green above and yellowish below with olive green and white banded sides and a red beak. It's home is central India and is believed to be endemic there. The red munia *Amandava amandava* is common in south eastern Asia. Another species of the genus amandava is said to be the Golden breasted waxbill *Amandava subflava* in Africa. The Avadavats are the only Weaver Finches to alternate between a colorful male nuptial plumage and a dull non-breeding plumage that resembles that of a female. Man introduced it from its southern and southeastern Asiatic homes to many tropical islands and also in Egypt.

These small-multicolored weaver finches evidently fulfill to an outstanding degree the human desire to care for and tend birds. So year after year they are caught in virtually unimaginable numbers to fill cages in many countries (Sane, 1978). There is no bird dealer who cannot offer some weaver finches. Therefore it may be assumed that such sustained catching of the birds in great numbers must have reduced their numbers in the wild. But the population of most weaver finches, in spite of their popularity as cage birds have not yet been reported as threatened. Recent considerations of the green munia as vulnerable (IUCN) and the report of black throated munia as uncommon (Satish,et al.2003) has brought in concern as the two species are the endemics of the family found in the country.

Most of the work on Estrildidae in India were related to their roles as agricultural pests.(Sharma et al. 1987., Varghese et al.1981., Bhargava,1999., Subramanya,1994).Practically no work has been done on the taxonomy and relationships of the species of munias found in the subcontinent.

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CHAPTER III

MATERIALS

3.1 THE MUNIA POPULATION

3.2 CHEMICALS AND BIOLOGICALS

3.3 GLASSWARE AND PLASTICWARE

3.4 EQUIPMENTS

3.5 RAPD PRIMERS

3. MATERIALS

3.1: THE MUNIA POPULATION.

Munias have a wide distribution in India with seven well known species (Ali.S; 1996). Of these, two belong to the genus *Estrilda;* the Red munia *E.amandava* , and the Green munia *E.Formosa,* or genus *Amandava* as they are usually referred to (*Amandava amandava* and *Amandava formosa*) being predominantly the Indian representatives of the genus *Estrilda*. They are also commonly called the Avadavats that seem to have arisen from the name of the north Indian city of Ahmadabad in Gujarat. The other five species belong to the genus *Lonchura;*the White backed munia, *Lonchura striata* the Black headed munia *Lonchura malacca,* the Spotted munia *Lonchura punctulata,*the White throated munia *Lonchura malabarica* and the Rufousbellied munia *Lonchura kelaarti* (Ali and Ripley 1973).

Two of the munia population considered here, the Green Munia and the Rufousbellied Munia are endemic having a restricted distribution in India; the former in the central part of the country and the latter in the Eastern and Western Ghats. The Green munia is already declared a vulnerable species. And the Rufousbellied munia or the Hill munia as they are commonly called was not procured in the present study due to its sparse and restricted distribution. The munias generally occur in grasslands, savannah type vegetation and forests. They are gregarious birds feeding mostly on grass seeds. The two species like *L. malacca* and *L. malabarica* do considerable damage to paddy. All species found in India are captured in large scale and sold as pets (Sane, 1978).

3.1.1: Red Munia or Avadavat . (Local name: Lal Munia.).

Plate: 3.1 ***Amandava amandava (1758).***

Description:

Sparrow size; length 10cm Breeding *Male* is brown and crimson coloured with white spots on wings, flangs and white tipped tail. *Female* is brown above and spotted on wings; crimson rump; dull white throat; buffy grey breast and yellow brown below. Non-breeding male is like the female with grayer throat and distinctive upper breast.

Habit:

They are essentially hill birds , being very common on the tops of the Nilgiris not being found on the plains. They associate in larger flocks than the other munias and are especially found where the hill guava bushes abound. They thrive well in confinement and are commonly seen in aviaries. They have shrill and high-pitched notes uttered in flights also. Generally feed on grass seeds but also insects when breeding.

Habitat:

Found among tall grass, reeds, sugarcane, scrub, and gardens in small flocks along with other munias all over India and in the south of Himalayan foot hills.

3.1.2: Green Munia. (Local Name: Hari Munia).

Plate: 3.2. ***Amandava formosa (1790).***

Description:

Size 10 cm Male: olive green above, with blackish tail ; yellow below; paler on throat; with prominent dark and white barrings on flanks that is diagnostic. Female: more brown above; paler below, with fewer barrings on flanks.

Habit:

They form small flocks sometimes gathering into several dozen birds. They do not usually associate with other munias. But like the other members of the family they feed on ground , picking up grass seeds and sometimes raids standing crops.The voice is a faint swee…..swee which changes when disturbed.

Habitat:

They are endemic with a restricted distribution in central India. Usually found from Rajasthan, Bihar , Maharashtra to Andhra in south. Located among grass and low bushes, tall grassland, sugarcane fields, in open deciduous forests and stony scrub jungle.

3.1.3: Black headed Munia (Local Name: Attachemban)

Plate: 3.3. ***Lonchura malacca (1766)***

Description:

Size: 10cm Sexes alike. A small chestnut , black and white munia with the typical short heavy grey conical bill. From above the head is black and rest of upper parts rufous-chestnut with a darker rump.Below the throat breast,center of belly, under tail coverts and thighs black. Rest of underparts white.

Habits:

Generally gregarious, except when breeding as in other munias. They are common in swampy low lying and ill drained areas with feathery grass and reeds, and the vicinity of wet paddy cultivation in forest. Feed on grass seed, paddy and ocassionaly on insects. They frequently associate with Ploceus manyar in the breeding season in south India. Feeds on the ground in undulating rabbles and move up into tree-tops when disturbed.

Habitat:

A permanent resident of the plains especially on the malabar coast. Like others of the genus, associates in small flocks being found chiefly in cultivated areas and on the banks of back waters. Found on marshlands, reedbeds grass fields and neighbourhood of flooded paddy cultivation in forest clearing

3.1.4: White rumped Munia. (Local Name: Attakaruppan.)

Plate: 3.5. *Lonchura striata (1766)*

Description:

Size:10 cm. Asmall black and white finch with a heavy bluish conical bill and wedge shaped tail.White rump and white abdomen conspicuous in flight.Sexes are alike. Found in groups in open cultivated country.

Habit:

Quite active and seen in small to medium sized flocks along other munias. Feeds on grass seeds crops and on insects. Feeds on the ground and also on standing crops and grasses. Sometimes large and mixed gatherings can cause damage to standing crops.

Habitat:

It is found on hills to a height of about 4800 feet and also on the plains of western India. It is seen rare not altogether absent in the east coast.

3.1.5. Spotted Munia (Local name : Chuttiatta)

Plate: 3.4. *Lonchura punctulata,(1758).*

Description:

Size:10 cm . Choclate brown above with faint pale shaft streaks below sides of head, head ,neck and lower throat is chestnut; chin and throat much darker. Brest and flanks speckled black and white. Sexes are alike.

Habit:

Are sociable and seen in flocks of upto hundredin non breeding season. Feeds on the ground and stems of rice or grass, on seeds small berries and also insects.

Habitat:

A common and widespread resident of the plains and hills. Though it does not ascend the hills of Travancore it is seen in the hills of Nilgiris.

3.1.6: White throated Munia. (Local Name: Vayalatta)

Plate:3.6. *Lonchura malabarica (1758)*

Description:

Size:10cm. A plain earthy brown thick billed little finch with painted back tail, white upper tail coverts and whitish under parts Sexes alikeFound to flock in dry open scrub country.

Habit:

Gregarious, and mostly keeps to scrub in open country, feeds on griound and on standing crop, especially millet. Overall a rather dull bird both in colour and demeanour. Affects gardens, scrub jungle etc. and is always seen in small flocks of a dozen or so individuals.

Habitat:

The commonest munia on the plains along the eastern side of the country. It is also found on the hills upto an elevation of 3000ft or so. In spite of its name it is rare on the Malabar coast.

3.1.7: Rufousbellied or Blackthroated Munia. (Local Name: Thottakaran)

Plate: 3.7. ***Lonchura kelaarti*** (1863).

Description:

Size:10 cm. Above forehead wings and tail is dark chocolate brown with pale shaft streaks. Below the cheeks throat and breast is blackish brown. Rest of the under parts is pinkish brown. Sexes are alike.

Habit:

Frequently found in mixed flocks with *L.striata,* but prefer higher and wetter country than *L..striata.* Affect scrub grassland fallow fields in forest clearings, neighbourhood of settlements and cultivation. Does not occur in dry scrub zone.

Habitat:

Endemic to the hills of south western India from Coorg and Southern Karataka through Kerala and western Tamilnadu to the Ashambu hills . Also a summer visitor at higher levels in the Nilgiris.

Of the seven species, the hill munia is the most uncommon with a very sparse distribution. They were last seen in large numbers during the bamboo flowering season in the foothills of Nilgiris. And for the past few years they have been reported uncommon.

3.2: CHEMICALS AND BIOLOGICALS.

All the chemicals used were of molecular biology grade or extra pure analytical reagent grade. Ethylene diamine tetra acetic acid (EDTA), chloroform , isoamyl alcohol, phenol, sodium dodecyl sulphate (SDS), sodium hydroxide were obtained from **SRL** Chemicals. Sodium chloride was obtained from **Qualigens**. Tris base, Tris–Cl, Proteinase–K, Agarose, Taq-DNA polymerase, dNTP mix from Messers **Promega.**

- ❖ Template (target DNA: about 10-100 ng)
- ❖ Oligonucleotides primers (5 μm). Available commercially from Operon Technologies Inc., Alameda, USA.
- ❖ Taq polymerase (5 U μl^{-1})
- ❖ Taq polymerase buffer (10 x)
- ❖ $MgCl_2$ (50 mM stock)
- ❖ dNTP (5 mM)
- ❖ Agarose- Normal and Large Fragment.
- ❖ 10x TBE buffer. pH 8.3 (10 mM Tris-borate, 1 mM EDTA)
- ❖ DNA Molecular marker standards
- ❖ Sterile Deionized Water (SDW)
- ❖ Gel loading buffer (0.04 % bromophenol blue, 0.04 % xylene cyanol FF, 5 % glycerol [in water])
- ❖ Ethidium bromide (0.5 m g m l^{-1})

3.3: GLASSWARE AND PLASTIC WARE.

Glassware were from either **Borosil** or **Schot-Duran**. Plasticware including multiwell plates were obtained from **Nunc, Falcon (BD); Greiner, TPP and Axygen.**

3.4: EQUIPMENTS.

All the Equipments for the analytical work was from Indian Institute of Spices Research Marykunnu, Kozhikode, Rajiv Gandhi Centre for Biotecnology Trivandrum and Indian Veterinary Research Institute Muktheswar .

- Programmable thermal cycler
- Electrophoresis power pack
- Agarose gel electrophoresis tanks, trays and combs
- UV transilluminator
- Darkroom
- Photographic equipment
- UV face shields
- Set of micropipettes
- Sterile microfuge tubes (1.5 and 0.5 ml)
- Sterile tubes for thermal cycler
- Sterile micropipette tips
- Ice bucket

3.5: RAPD PRIMERS.

30 Primers of 10 base pairs each were obtained from **Operon Technologies Inc., Alameda, USA.** and tested. Those resulting in visible, reproducible and easily scorable bands were selected. A total of only 11 decamer oligonucleotide primers was selected to investigate the six *Estrildidae* species.

Table.1: List of names and the sequence data of the Random Primers used in RAPD Analysis.

No.	**Name**	**Nucleotide Sequence**
1.	BG 81	**5'**- T G C C G A G C T T - **3'**
2.	BG 51	**5'**- A G C C G T C C T C -**3'**
3.	3/134	**5'**- C A G G C C C T T C - **3'**
4.	8/101	**5'**- G A A A C G G G T G -**3'**
5.	5/134	**5'**- A A T C G G G C T G -**3'**
6.	7/101	**5'**- A G G G G T C T T G - **3'**
7.	6/134	**5'**- C AG C A G C C A C - **3'**
8.	4/134	**5'**- T G C C G A G T G G -**3'**
9.	1/101	**5'**- G T G A T C G C A G -**3'**
10.	7/134	**5'**- T C T G T G C T G G - **3'**
11.	6/101	**5'**- T C G G C G A T A G- **3'**

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CHAPTER IV

METHODS

4.1 POPULATION SAMPLES

4.2 ISOLATION OF GENOMIC DNA

4.3 DNA QUANTIFICATION

4.4 QUALITATIVE ANALYSIS OF DNA

4.5 RAPD ANALYSIS OF MUNIA GENOME

4. METHODS.

4.1: POPULATION SAMPLES

Population genetic structure of six species of munias in India were assessed from the analysis of 60 samples, i.e., 10 individuals collected from each of the six species of munia populations; *Amandava amandava*, *Amandava formosa*, *Lonchura malacca*, , *Lonchura striata*, *Lonchura punctulata* and *Lonchura malabarica*. Genus *Amandava(Estrilida)* include the true wax bills, and are mostly colourful whereas genus *Lonchura* include the mannikins that are somber coloured. *A. Formosa*, the green munia found only in northern India is an endemic species. The seventh species also an endemic species *L.kelaarti*, due to its very restricted distribution was not procured and therefore not included in the present study. The samples were mostly captured from Nilambur KFRI premises and also obtained from different pet shops in and out of Kerala. Care was taken to obtain individuals of a species from different flocks.

4.2: ISOLATION OF GENOMIC **DNA.**

DNA was extracted from two different tissues i.e. blood and liver. Since isolation of DNA from liver involved sacrifices of the bird the other tissue was chosen for the study. However a comparison of the two tissues were done to show that DNA from blood tissue is more intact than from liver tissue.

DNA was isolated from 0.5ml of venous blood collected by cardiac puncture using A26 gauge needle. The syringe was heparinised before

collection of samples. After mixing well with the anticoagulant the blood samples were collected and stored at 4° C.

DNA was isolated essentially as per the salting out procedure of Miller et al 1988 with slight modification. The total cells from the samples were separated by centrifugation at 7000 rpm for 5min in a microfuge.

The cell pellet was re suspended in 200µl of T.E buffer (pH 7.6) and added with 600µl of lyses-buffer and kept at 4° C for five minutes. The solution was spun at 12000 rpm to sediment all the nuclei. 100µl of T.E was added to mix with the nuclear pellet then added the proteinase-k at a concentration of 20µg/ml and SDS to a final concentration of 1%. The solution was incubated at 56 degree Celsius for one hour. Then approximately 1/3 volume of 5M NaCl was added and mixed thoroughly well using a pipette. Then kept the mixture for 5 min at room temp. Equal volume of chloroform: isoamyl alcohol (24:1) was added and vortexed for five minutes. The solution was centrifuged at 7000rpm. for 10 min to separate the organic and aqueous solvent phases.

The upper layer containing the DNA in aqueous phase was carefully removed into another tube taking care not to disrupt the interface precipitate. Two volumes of chilled ethanol were added and gently inverted many times. The precipitated DNA was removed with a micropipette and transferred to a 1.5 ml microfuge tube. The DNA pellet was washed with 70% ethanol and partially air-dried. Depending on the quantity of precipitated DNA it was resuspended in 100-400ml of T.E buffer. The solution was kept overnight at 56 degrees Celsius to facilitate the dissolution process. The solution was then kept stored at 4 °c.

4.3: **DNA** QUANTIFICATION.

Following extraction DNA was quantified and checked for its purity and RNA-protein contamination by the spectrophotometry method. 1ml of dissolved DNA was made upto 100ml with milli-Q water in the quartz microcuvette. The absorbance OD of the diluted DNA solution was taken at both 260 and 280 nm wave lengths using a Schimatsu UV spectrophotometer. The ratio of absorbance 260-280 was taken as the index of purity. DNA samples that gave a ratio between 1.6 – 1.9 were directly used for further analysis whereas other DNA samples were purified further using phenol chloroform extraction method.

Samples were further purified by adding with equal quantities of tri saturated phenol and the mixture was vortexed for 5 min and centrifuged using a microfuge for 5 min and the upper phase was carefully transferred to another tube. Equal quantities of phenol and chloroform isoamyl (24:1) alcohol were added and vertexed for 5min for centrifugation.The upper phase was again re extracted with chloroform once and the DNA was ppted using two volumes of absolute alcohol. The DNA pellet was washed at least twice with 70% alcohol air dried and dissolved in T.E as described above. Taking the ratio of absorbance at 260nm and 280nm (260/280) respectively estimated the quantity of genomic DNA. A ratio between 1.7 –1.8 indicated good quality DNA without protein contamination. DNA quantification was done according to the following formula:

1 O.D at 260nm = 50 ng of DNA.

The O.D of each DNA sample at 260nm was measured and quantified accordingly. . The purity and concentration of the different samples of each species are given in **Table - 3.** After adjusting the concentration of all the samples to 500 ng/ml an aliquot of the samples was preserved in 70% alcohol for long -term storage at -20 °c.

Table.3: **Spectroscopic Analysis** of DNA for checking the Purity and Concentration of the selected six individual DNA samples from each of the six species of the munia genome. Each letter stands for each Species.

R– Red munia; **G**– Green munia ; **B**– Blackheaded munia;

No.	**Samples**	**A_1**	**A_2**	**A_1/A_2**	**DNA conc. Ψ**
1.	R_1	1.458	0.857	1.7154	67.746
2.	R_2	2.323	1.432	1.6294	108.24
3.	R_3	1.102	0.608	1.8299	51.398
4.	R_4	1.922	1.098	1.7599	90.038
5.	R_5	2.683	1.446	1.8735	125.42
6.	R_6	0.744	0.416	1.6106	35.050
7.	G_1	3.436	1.979	1.7791	156.86
8.	G_2	4.000	2.466	1.6595	181.44
9.	G_3	3.311	2.173	1.5273	154.4
10.	G_4	3.215	1.959	1.6565	148.92
11.	G_5	3.135	2.045	1.5349	146.54
12.	G_6	3.215	2.737	1.1754	147.67
13.	B_1	2.045	1.093	1.8709	96.590
14.	B_2	2.683	1.423	1.8862	126.80
15.	B_3	1.391	0.774	1.7978	65.622
16.	B_4	2.834	1.573	1.8023	133.68
17.	B_5	0.945	0.485	1.9491	44.716
18.	B_6	1.256	0.661	1.9004	126.16

A_1 - OD 260 **A_2** - OD 280 Ψ - μg / ml at 1 / 20 dilution.

Table.3. Spectroscopic Analysis of DNA for checking the Purity and Conc.
Contd.: of the selected six individual DNA samples from each of the six species of the munia genome. Each letter stands for each Species.

W– Whitebacked munia; **S**– Spotted munia; **T**– Whitethroated munia.

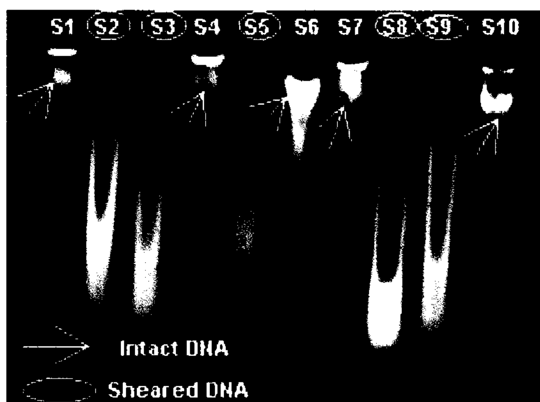
No.	Samples	A_1	A_2	A_1/A_2	DNA conc. Ψ
19.	W_1	3.612	1.909	1.9512	165.34
20.	W_2	2.591	1.418	1.832	121.99
21.	W_3	1.591	0.862	1.8454	75.109
22.	W_4	1.178	0.605	1.9464	55.744
23.	W_5	0.468	0.235	1.9927	22.151
24.	W_6	1.361	0.810	1.6767	64.159
25.	S_1	1.292	0.694	1.8614	61.010
26.	S_2	0.419	0.238	1.7603	19.725
27.	S_3	0.081	0.052	1.5457	37.744
28.	S_4	0.096	0.061	1.5772	45.050
29.	S_5	1.106	0.616	1.7938	52.140
30.	S_6	1.900	0.014	1.8743	89.769
31.	T_1	2.121	1.177	1.8106	99.424
32.	T_2	2.635	1.478	1.7826	124.32
33.	T_3	2.223	1.326	1.6765	104.58
34.	T_4	2.683	1.666	1.6056	126.35
35.	T_5	2.591	1.418	1.8312	121.99
36.	T_6	4.0000	2.613	1.5564	181.83

A_1 - OD 260 A_2 - OD 280 Ψ - µg / ml at 1 / 20 dilution.

4.4: QUALITATIVE ANALYSIS OF **DNA** IN AGAROSE GEL.

The degradation of the DNA were assessed by electrophoresis on 0.8% agarose gel as per the standard protocol. (Sambrook et al 1989). 0.2g of low EEO agarose (Messers Promega) was added to 25ml of 1x Tris borate EDTA (TBE Buffer) and melted in a microwave oven for 1 min. After cooling down to approx. 50° Centigrade Ethidium bromide was added at the rate of 0.5μg/ml. The molten agarose was poured into a pre cast gel tray with a comb. After solidifying the gel tray was immersed in the electrophoretic tank containing 1x TBE buffer. 1 μl of the DNA samle was mixed with 4μl of the 1x TBE buffer and 1μl of 6x loading dye in a parafilm sheet and loaded on the gel. Electrophoresis was carried out at 100 volt for 1 hr. The DNA samples were visualized in the gel using a UV transilluminator.

Plate: 4.1. Qualitative analysis of DNA by Agarose Gel electrophoresis. The sheared DNA samples were discarded.



4.5: **RAPD** ANALYSIS OF MUNIA GENOME.

With the advent of PCR technology many novel techniques were evolved for detecting random genetic variation between groups of organisms. One if the simplest PCR based methodology for bringing out random genetic variation is RAPD (Random Amplified Polymorphic DNA). William et al (1990) has shown that when oligos of smaller size of about 10 nucleotides are used as primers in genomic DNA at lower annealing temperature then these oligos can bind at different regions of both the strands of DNA in both the directions. This random binding of the primer can result in a number of PCR products of different sizes from individual genomes. Comparison of these strands between related taxas can be used for generating data on genetic identity.

Even though there have been methodological and analytical controversies surrounding the use of RAPDs to estimate the genetic variability many studies have been conducted indicating the usefulness of RAPD in assessing genetic variations within and between

In order to compare the genetic variation at DNA levels among the different groups of munia, the RAPD method of DNA fingerprinting was attempted. RAPD approach was used for generating random fingerprints of different species of munias. The random stretches of DNA were amplified with different sets of oligonucleotide primers in PCR reactions. A total of 30 different primers ranging from AA to AZ of the operon technologies USA were screened for their efficacies in generating fingerprints in munia genome.

Based on the presence of clear and distinct banding patterns, 11 of the 30 random primers screened were selected to analyze the 36 intact DNA samples obtained from the six species of munia genome.

4.5.3: Procedure

A typical RAPD reaction involves primarily the preparation of the reaction mix containing all but the variable component, which can then be put into the individual tubes. For example, for the 25 μl final reaction volume PCR of 36 different samples of template DNA the following mix was prepared on ice:

4.5.3.1: Reaction mix of 950μl for 38 PCRs prepared for the 36 different target DNAs.

- Water 657 μl
- 10_x PCR buffer (no Mg^{2+}) 95 μl
- 25mM $MgCl_2$ 95 μl
- 10 mM dNTPs 19 μl
- 5 pmol Primer 38 μl
- 5 U μl^{-1} Taq polymerase 8 μl
- Total volume 912 μl
- Dispensed @24μl/tube in 36 tubes.
- Added 30-35ng Template DNA 1μl per tube.

4.5.3.2: The Polymerase Chain Reaction.

RAPD amplification (AP PCR) reactions were thus performed in 25 μl final volumes containing 30 ng of DNA in 10mM Tris (pH 8.3), 50mM KCl, 2.0 mM $MgCl_2$, 100mM of each dNTPs, 20 picomol of a 10 base pair random primer and 0.5 U of Taq polymerase and water. After mixing well the contents were spinned down and capped with a layer of mineral oil to

prevent evaporation loss. The amplifications were carried out in an eppendoff thermocycler with a 2-mins initial denaturation step at 94 °C followed by 45 cycles of 1 min denaturation at 94 °C, 1 min annealing at 33 °C and 2 mins extension at 72 °C. To check the reaction, amplified RAPD products stained with ethidium bromide were mixed with 0.5 volumes of a loading dye solution and run on a 0.8 % agarose gel electrophoresis at 100v for 1 hour and visualized under UV light. Best amplified products were thus chosen, added a drop of T.E. and stored upto a week for running the final cosmetic gel to score the banding pattern. A 2% cosmetic gel for electrophoresis was prepared by a 3:1 composition of Large Fragment agarose and normal agarose to run the well amplified RAPD products at 70volts for 3-4 hours. The products were then run alongside a 100bp DNA ladder (molecular weight standard) and visualized for the analysis of bands.

A total of 60 different primers ranging from AA to AZ of the operon technologies USA were screened for their efficacies in generating fingerprints in munia genome. Based on the clear and distinct banding 11 of the 60 random primers screened were selected to analyze inter population variance using pooled DNAs of the six species. i.e. DNA samples of individuals of a species population were pooled to represent that population. The six species specific pooled DNAs were taken for the estimation of interpopulation variance. The same selected primers were then used to analyze the intra population variance among individuals within each species. For every individual sample, the bands were scored as present (X) or absent (0) in a presence / absence matrix for estimating genetic dissimilarity between populations of each species and between individuals of each species population.

4.5.3.3: Trouble shooting in the RAPD-PCR reactions.

The following problems were encountered when carrying out RAPD-PCR and it was important to bear in mind the following:

a. Since varying the concentration of DNA can alter the number of bands for munia genome about 30-35 ng DNA was per reaction to produce reliable and consistent fragments.
b. PCR buffer (10x) usually provided by Taq polymerase suppliers may or may not contain Mg^{2+} ions. So RAPD profiles was found to vary depending on the Mg^{2+} ions concentration.
c. There are different sources of Taq polymerase and there was great variation between profiles produced by Taq polymerase from different companies. Both the Taq polymerase and the concentration required was determined experimentally.
d. Though thermal cycler programme above was recommended there are a large number of alternative cycling times and temperatures that are equally important depending on the machine and the PCR tubes wall thickness.

Generally when RAPD-PCR did not work there was likely to be something wrong with the template DNA, primers, Taq polymerase or choice of conditions. Initially it was important to try and repeat the PCR under the same conditions to ensure that there was not a simple error that resulted in the failure. In addition it was recommended to include a positive and negative controls. A positive control with a template known to amplify well will ensure that all reagents are added and that they are all functioning. A negative control without template DNA will reveal any contamination. In most cases when the PCR did not work and it was not clear what might be causing the problem, it was worth starting from the beginning by disposing of all the reagents used and preparing fresh ones.

4.5.3.4: Factors that affected the reproducibility of RAPD profiles

Several factors were found to affect the number, size and intensity of bands. These included PCR buffers, dNTPs, Mg^{2+} concentration, cycling parameters, source of Taq polymerase, condition and concentration of DNA and primer concentration. Results obtained by RAPDs were highly prone to user error and bands obtained varied considerably between different runs of the same sample. This limitation was, however, easy to overcome through practice and many articles have reported consistency in the profiles obtained from different runs of the same sample. The problem of the reproducibility of RAPDs data between laboratories that use different PCR machines may soon disappear with the availability of new improved PCR machines on the market. Some have reported identical banding patterns obtained using different thermocyclers and success depends mainly on a defined set of conditions, which should be maintained constant when using different machines.

4.5.4: Data analysis

Data from molecular marker techniques require detailed analysis to establish genetic relations. For each primer, polymorphic bands are scored for their presence or absence in all the landraces and/or individuals by visually assessing photographs of the gels. It was important that duplicate amplifications are performed to confirm the reliability of the bands. Amplification products of all individuals and/or landraces are listed as discrete character states in a present (1) or absent (0) matrix.

RAPD polymorphisms were analyzed with the assumption that bands from different loci do not co-migrate. Then levels of within population genetic variation were assessed by the number of unique band, percentage polymorphism and mean percentage band sharing. A band was considered

unique if it was detected only in a single population. Dividing the number of bands that exhibited polymorphism within a population by the total number of bands scored among all populations and then multiplying by 100 calculated percentage polymorphism.

Mean genetic diversity (D) was calculated as the average of pairwise relative distances (d) within populations. Pairwise relative distances between any two individuals were estimated using the following equation:

$$d = 1 - N_{XY} / N$$

where N_{XY} is the number of bands shared by the individuals Xand Y, and N is the number of total polymorphic sites. The proportion of polymorphic bands (P) was estimated for each population as the number of polymorphic bands divided by the total number of markers analysed.

Genetic variability was also estimated using the percentage of monomorphic (M) and the percentage of polymorphic bands (P) within populations.

The proportion of polymorphic bands (P) was estimated for each population as the number of polymorphic bands divided by the total number of markers analysed.

The genetic distance between any two populations was estimated by averaging D-values between pairwise comparisons from individuals of these populations (Gilbert et al, 1990).

Genetic distances were used in UPGMA cluster analysis with the soft ware program.Based on the UPGMA procedure, a genealogical dendrogram is constructed, based on values for inter-specific distances within the Estrildidae.

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CHAPTER V

RESULTS

5.1 BANDING PATTERN AND POLYMORPHISM

5.2 GENETIC VARIATION WITHIN SPECIES

5.3 GENETIC VARIATION BETWEEN SPECIES

5.4 GENETIC RELATIONSHIPS WITHIN ESTRILDIDAE

5. RESULTS

From the 11 RAPD primers a total of 61 polymorphic bands were scored between the species. PCR replicates showed repeatable banding patterns. When analyzing amplified products from two separate .albeit identical reactions on contiguous lanes all primers produced reproducible results. Bands that were not consistently amplified in the replicates were not considered. Each of the random primers produced distinct polymorphic banding patterns in all of the species examined. Every population sampled for interspecific variance had a genetically unique RAPD pattern.

5.1. BANDING PATTERN AND POLYMORPHISM.

The size of the amplified products ranged from 500–3000 bp, with 3 – 10 polymorphic bands per primer.The average number of bands per primer was 5. The six species studied showed that on an average only 16 % of the RAPD markers were monomorphic.

Figure.5.1: Banding pattern obtained for the 11 oligonucleotide Operon Primers.

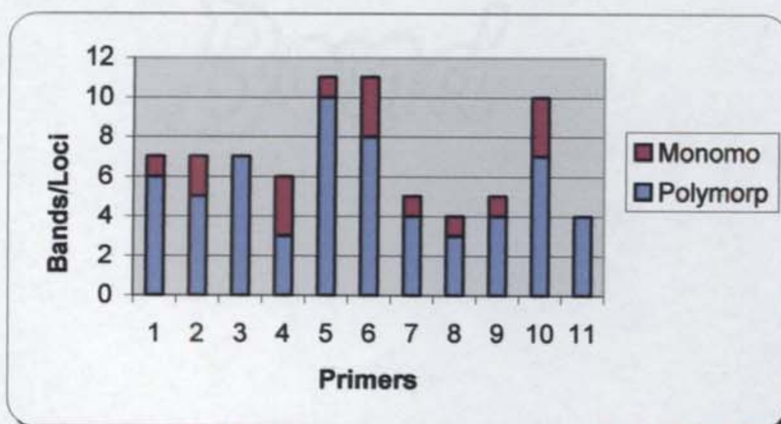


Table.5.1 : Number of **S**cored, **U**seful, **P**olymorphic and **M**onomorphic **Bands** obtained in the RAPD analysis.

No.	Name	Scored	Useful	Polymorphic	Monomorphic
1.	BG 81	14	8	6	2
2.	BG 51	9	7	5	2
3.	3/134	11	7	7	0
4.	8/101	12	6	3	3
5.	5/134	13	11	10	1
6.	7/101	11	11	8	3
7.	6/134	11	5	4	1
8.	4/134	6	4	3	1
9.	1/101	9	5	4	1
10.	7/134	10	10	7	3
11.	6/101	6	4	4	0

The banding pattern in the RAPD analysis data was calculated using Gene Tool software of Genegenius from **Syngene.** The profile analysis was done based on the molecular weight of each band amplified along a track run on the agarose gel. The Gene tool software was used for the detection of the visible amplified products and for the detection of the banding pattern .

The Gene snap software of the Gene genius from Syngene gave sharp images of the agarose gel that could be stored as data for further analysis by the Gene tool softwares.

PLATE.5.1: RAPD Banding

A. Example of RAPD markers amplified using Operon primer 10/134 in the six species of Estrildidae. Track M is the standard molecular weight marker and lanes 1-6 indicate the the six species of Munias.

Arrows indicate the polymorphic bands scored and used in the RAPD analysis.

B. The image of the RAPD band matching analysis between the six species with the Gene tool software program of Genegenius from Syngene

Track 6 is taken as the reference track.

5.2: GENETIC VARIATION WITHIN SPECIES.

The individuals sampled within each species for the estimation of intra-specific variance ranged from 6 to 10, with genetic variation not very significant. Within individuals of a species the number of polymorphic bands ranged from 5 to 10 with the three chosen primers that gave visible products. Of the 11 chosen primers, primer no. 5, 6, and 10 gave best banding results.

The number of bands obtained in each species are shown in the table 5.1. Six individuals were sampled from each species for the intraspecific variance analysis. The species which showed the greatest number of polymorphic bands was *A. Formosa* and the species which showed the least amount of genetic variation was *L. malabarica.*

Of the 61 polymorphic bands 12 were common for *A. amandava* and *A. formosa* indicating similarity in their genetic structure. The results obtained by the analysis of 26 loci showed that *L.striata* and *L.punctulata* had entirely the same genetic structure reflecting their extremely low genetic differentiation.

The levels of within population genetic variation as measured by the percentage analysis of polymorphic and monomorphic markers (Fig: 5.16 and 5.17) were similar for the six species of munias .

No unique bandings was detected in *A. Formosa* (Table.5.) while three were detected in *L.striata,* two in *L.malacca* and and one each in *A.amandava, L. punctulata and L. malabarica.* RAPD analysis also revealed putative species-specific amplified products. One such band was observed in the species *Amandavaa amandava.*

The polymorphic bands detected in the individuals of each munia species are depicted in the table 5.10 The proportion of polymorphic loci as in table 5.11.of the Estrildidae ranged from 0.026 to 0.048 and the overall mean of all species was 0.059 which was slightly low for the Passeriforms.

TABLE: 5.2. Banding Pattern obtained with RAPD Primers showing the Amplified Polymorphic bands between individuals in each Munia species

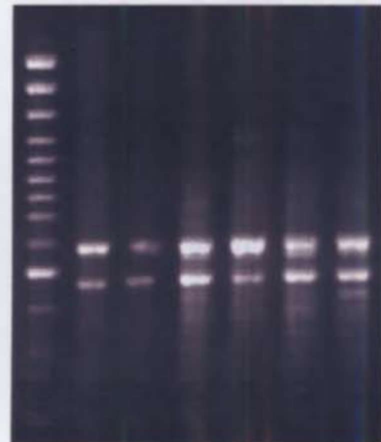
Species	P.bands	Indi. 1	Indi. 2	Indi. 3	Indi. 4	Indi. 5	Indi. 6
Red M	1	1	0	1	0	0	0
	2	0	0	1	0	0	1
	3	0	0	1	1	0	1
	3	0	1	1	0	0	1
	4	1	0	0	1	0	1
	5	0	1	0	0	1	1
	6	1	0	0	0	1	1
	7	0	0	1	0	1	1
	8	0	0	1	0	1	1
	9	0	0	0	1	1	1
Green M.	1	1	0	1	0	1	0
	2	1	1	0	0	1	0
	3	0	1	1	0	0	1
	4	0	1	1	0	0	1
	5	0	0	0	1	1	1
Black headed	1	1	1	0	0	0	0
	2	1	0	0	0	0	0
	3	1	0	0	0	0	1
	4	0	0	0	1	1	1
	5	0	0	0	0	0	1
	6	0	1	0	0	1	1
	7	1	0	0	0	0	1
	8	0	0	1	0	1	1
	9	1	0	0	1	0	1
	10	1	0	0	0	1	1
	11	1	1	0	0	0	1
	12	0	0	0	0	1	1

TABLE: 5.2.Contd. Banding Pattern of the 11- RAPD Primers showing the Amplified Polymorphic bands between Individuals in each Munia species.

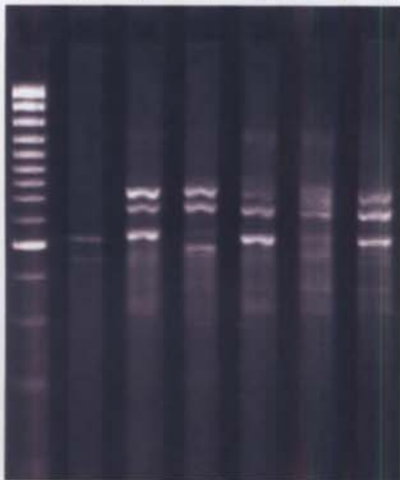
Species	P.bands	Indi. 1	Indi. 2	Indi. 3	Indi. 4	Indi. 5	Indi. 6
White rumped	1	1	0	0	0	0	0
	2	1	0	0	0	0	1
	3	0	0	0	0	0	1
	4	0	0	0	1	0	1
	5	1	0	1	0	0	1
	6	0	1	1	0	0	1
	7	0	0	0	0	0	1
Spotted	1	1	1	0	0	1	0
	2	1	1	1	0	0	0
	3	1	0	0	0	0	0
	4	0	0	0	0	1	1
	5	0	0	0	0	0	1
White throated	1	1	1	0	0	0	0
	2	1	0	0	0	0	0
	3	1	0	1	0	0	1
	4	0	1	0	1	0	1
	5	1	0	1	0	0	1

PLATE.5.2: RAPD Banding in *E.amandava.*

Primer – 4



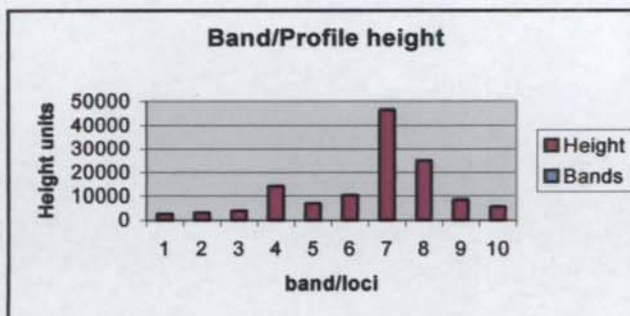
Primer – 3

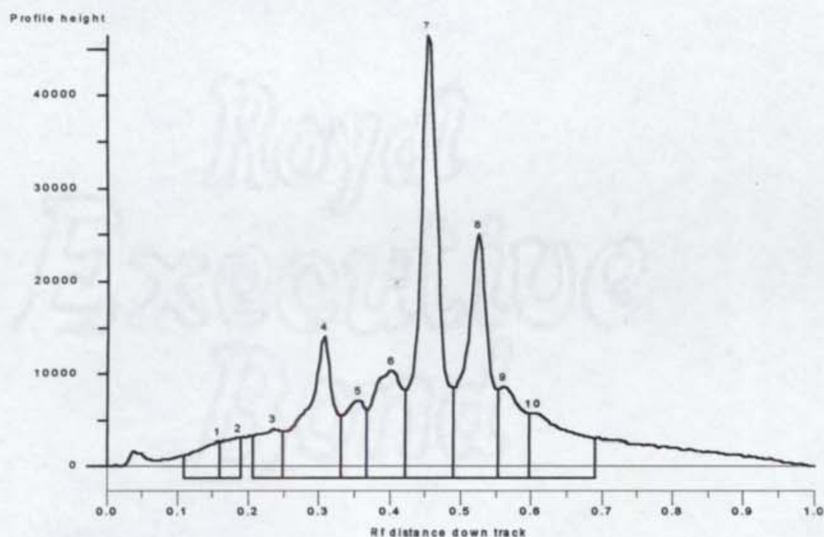


Primer – 9

Figure.5.2: RAPD Profile analysis of each track to identify Polymorphic bands in each individual of *E.amandava* species

Bands	Height
1	2712.564
2	3143.786
3	4050.598
4	14037.966
5	7143.661
6	10446.189
7	46406.379
8	24982.904
9	8619.617
10	5772.716





Bands	Raw vol.
1	787533.88
2	656624
3	1197694.8
4	4543093
5	1767105.6
6	3764775.8
7	11198635
8	6914363
9	2543265
10	2973614

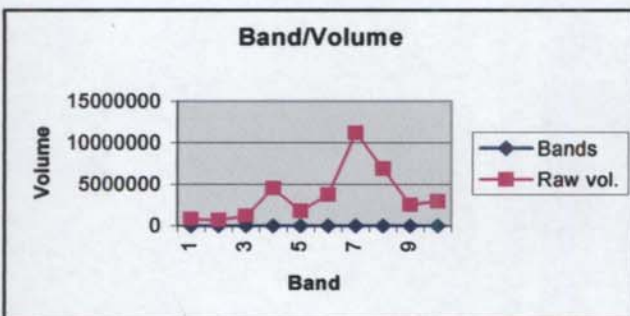
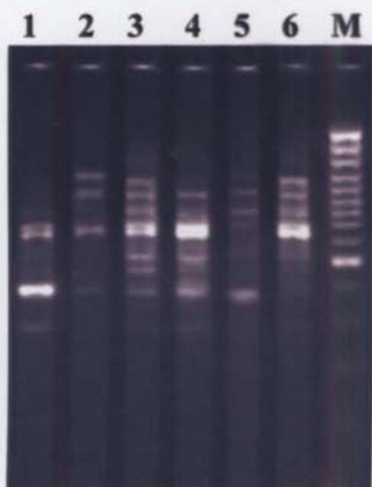
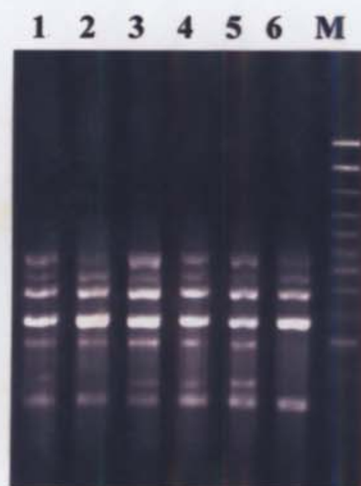


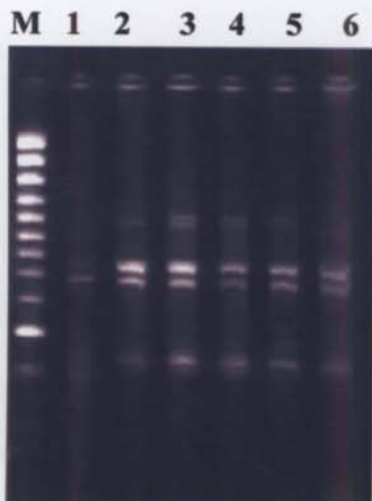
PLATE 5.3: RAPD Banding in *E. formosa.*



Primer – 4



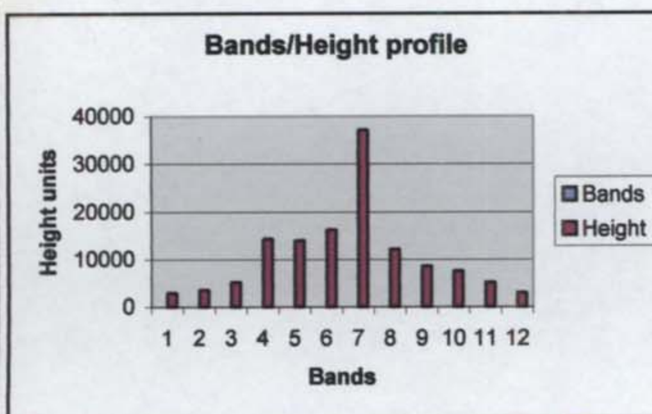
Primer - 3

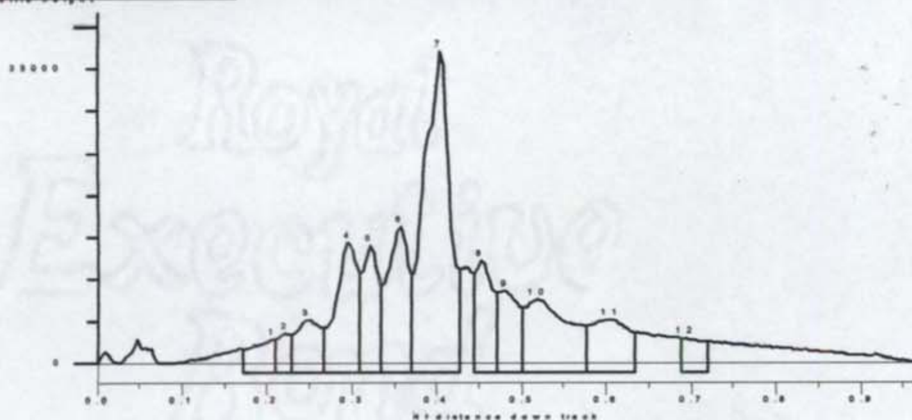


Primer – 9

Figure.5.3: RAPD Profile analysis of each track to identify Polymorphic bands in each individual of *E.formosa* species

Bands	Height
1	2907.68
2	3573.223
3	5191.277
4	14309.93
5	14015.3
6	16263.388
7	37085.219
8	12135.527
9	8600.204
10	7543.833
11	5203.127
12	2943.028





Bands	Raw vol.
1	672797.06
2	507027.56
3	1359044.1
4	3175670.5
5	2366901.5
6	3540917.8
7	10199857
8	2346450.5
9	1774445.8
10	3560221
11	2092564.1
12	680602.63

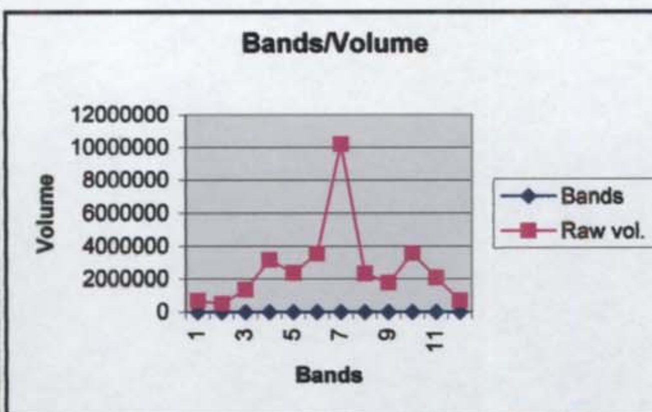
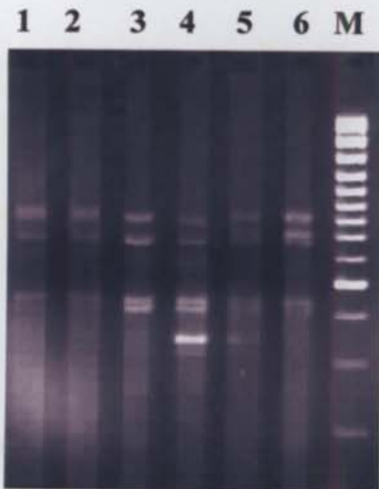


PLATE. 5.4: RAPD Banding in *L. malacca.*

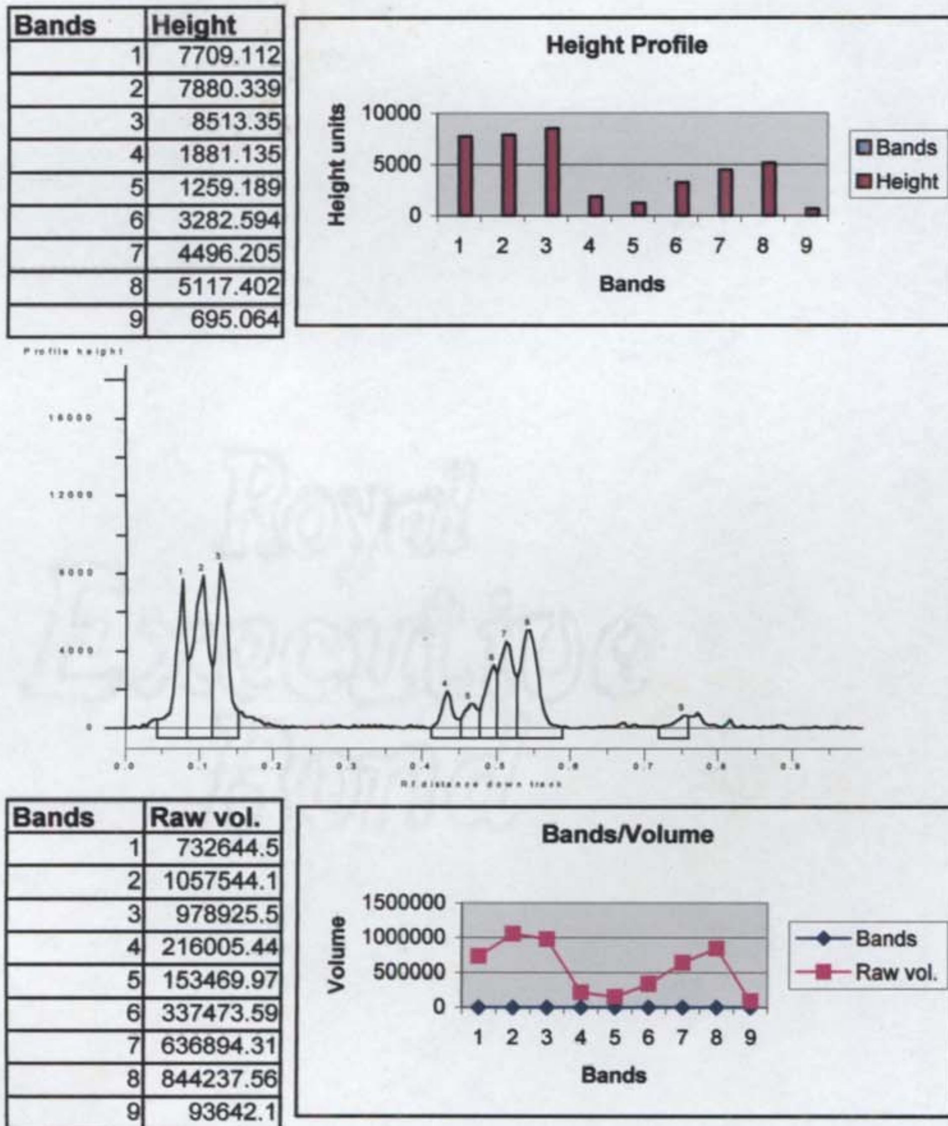
Primer – 4

Primer - 3



Primer – 9

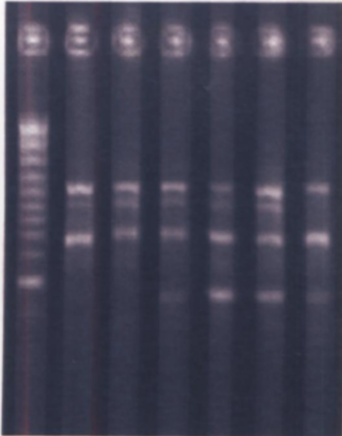
Figure.5.4: RAPD Profile analysis of each track to identify Polymorphic bands in each individual of *L.malacca* species.



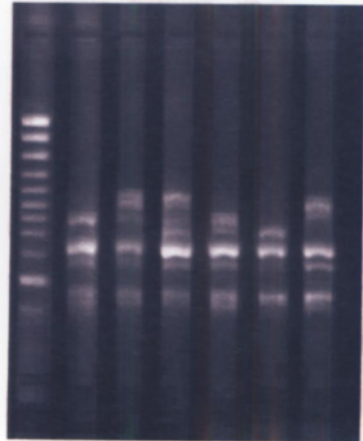
Bands	Height
1	7709.112
2	7880.339
3	8513.35
4	1881.135
5	1259.189
6	3282.594
7	4496.205
8	5117.402
9	695.064

Bands	Raw vol.
1	732644.5
2	1057544.1
3	978925.5
4	216005.44
5	153469.97
6	337473.59
7	636894.31
8	844237.56
9	93642.1

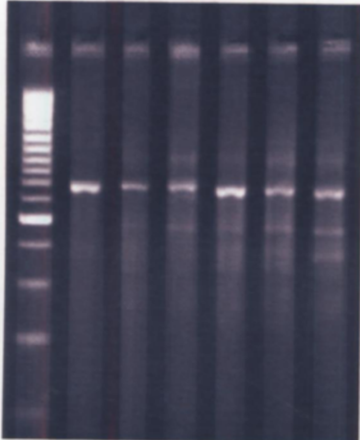
PLATE.5.5 RAPD Banding in *L.striata.*



Primer – 4

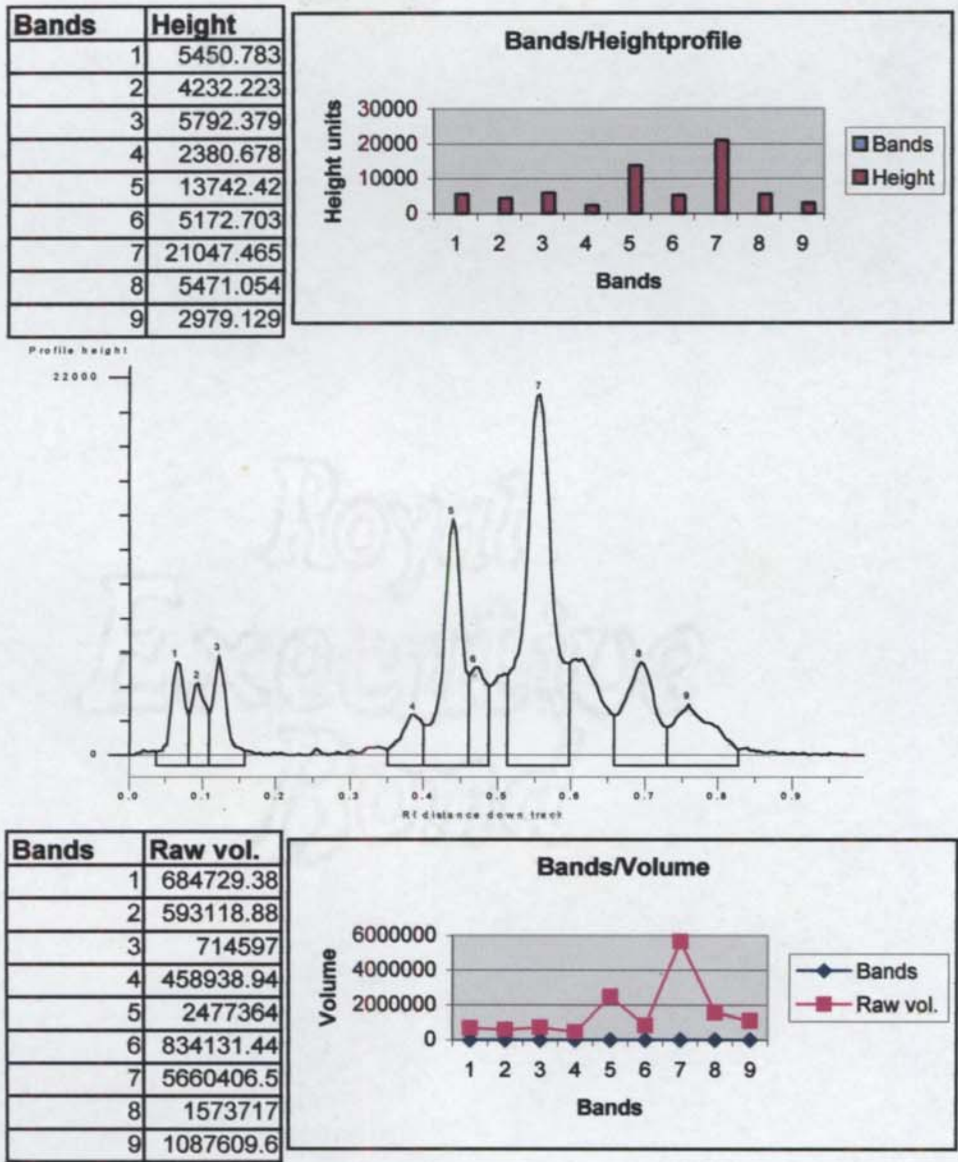


Primer - 3



Primer – 9

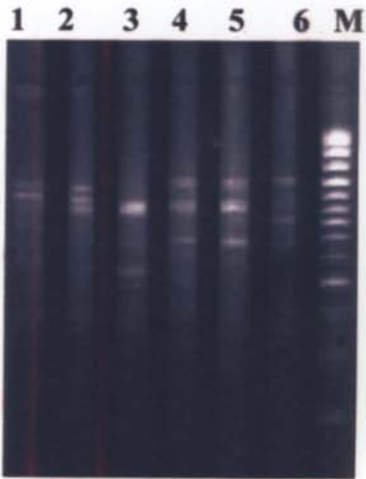
Figure.5.5: RAPD Profile analysis of each track to identify Polymorphic bands in each individual of *L.striata* species.



Bands	Height
1	5450.783
2	4232.223
3	5792.379
4	2380.678
5	13742.42
6	5172.703
7	21047.465
8	5471.054
9	2979.129

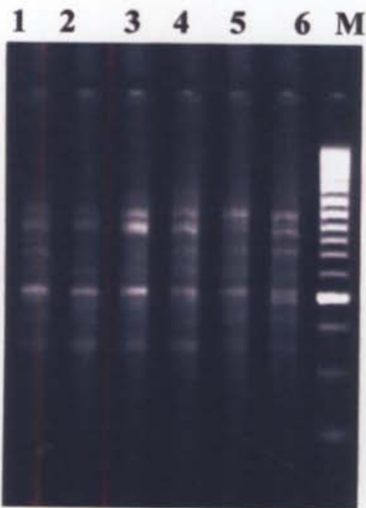
Bands	Raw vol.
1	684729.38
2	593118.88
3	714597
4	458938.94
5	2477364
6	834131.44
7	5660406.5
8	1573717
9	1087609.6

PLATE.5.6: RAPD Banding in *L.punctulata*.



Primer – 4

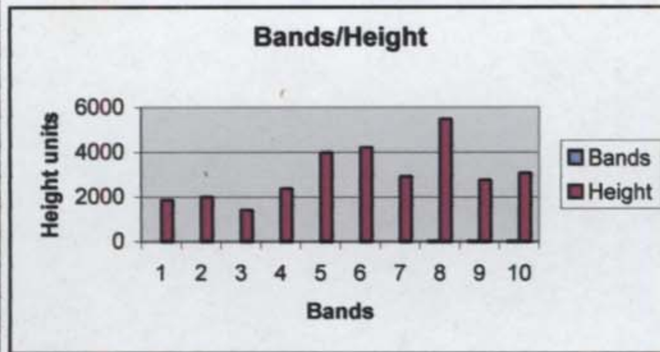
Primer - 3

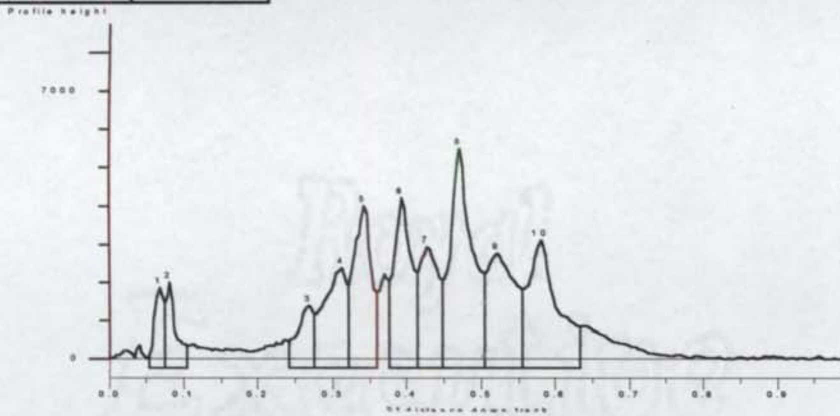


Primer - 9

Figure.5.6: RAPD Profile analysis of each track to identify Polymorphic bands in each individual of *L.punctulata* species.

Bands	Height
1	1855.734
2	2000.212
3	1393.036
4	2373.971
5	3980.408
6	4194.365
7	2928.16
8	5488.34
9	2755.9
10	3084.479





Bands	Raw vol.
1	176817.72
2	207087.44
3	228305.97
4	601288.38
5	757639.25
6	795735.94
7	610255.06
8	1348986.1
9	834423.25
10	970062.75

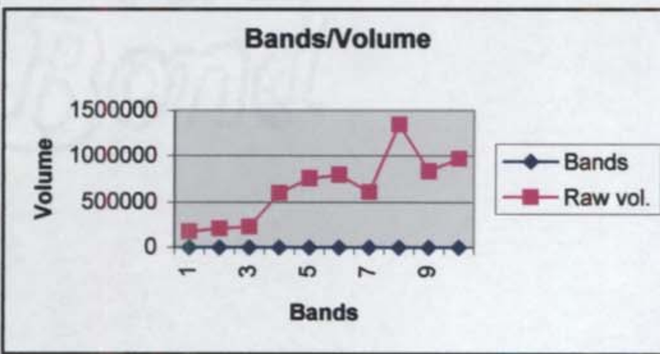
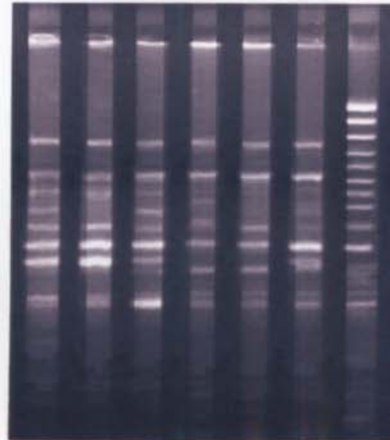


PLATE.5.7: RAPD Banding in *L. malabarica.*

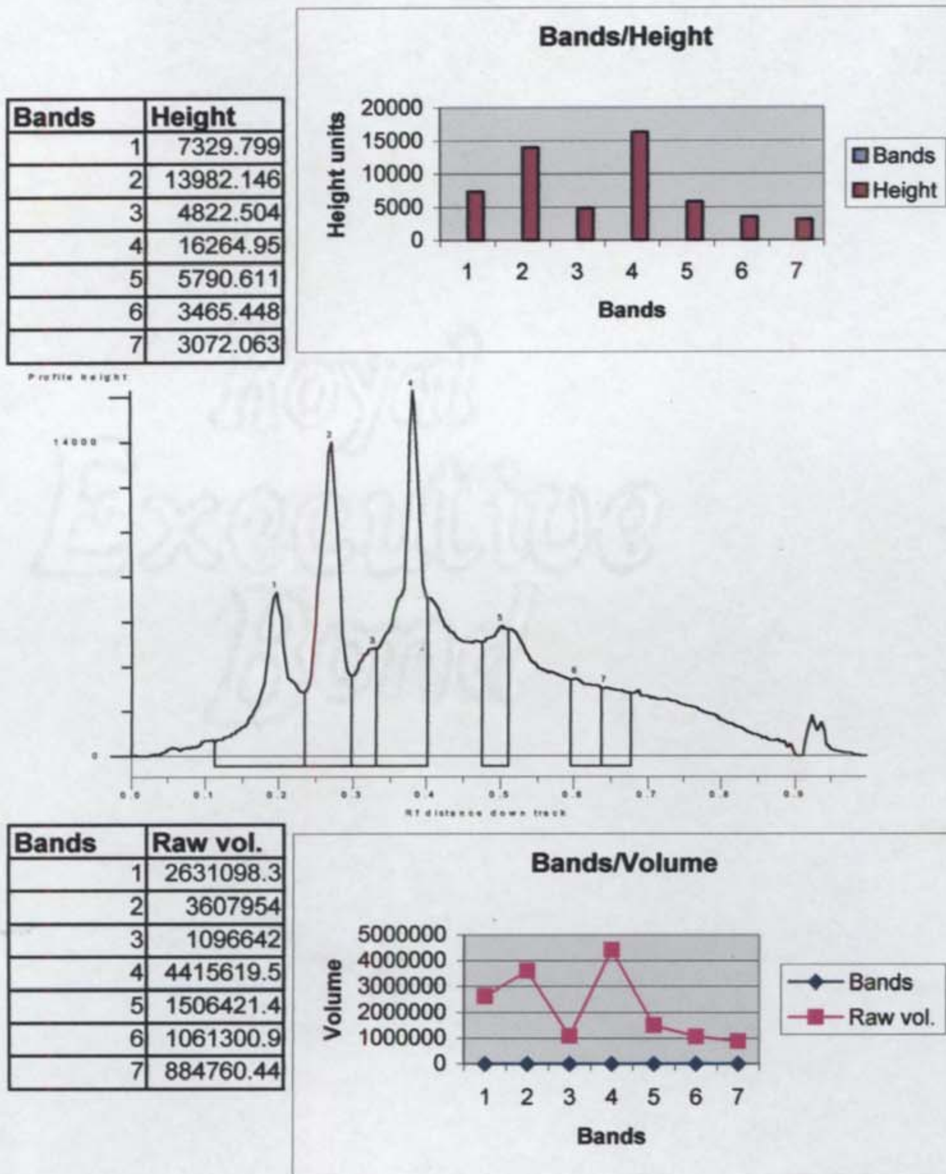
Primer – 4



Primer - 3

Primer - 9

Figure.5.7: RAPD Profile analysis of each track to identify Polymorphic bands in each individual of *L.malabarica* species.



Bands	Height
1	7329.799
2	13982.146
3	4822.504
4	16264.95
5	5790.611
6	3465.448
7	3072.063

Bands	Raw vol.
1	2631098.3
2	3607954
3	1096642
4	4415619.5
5	1506421.4
6	1061300.9
7	884760.44

Mean genetic distances (D) was calculated as the average of pairwise relative distances (d) within populations. Pairwise relative distances or the matching coefficients between any two individuals were estimated (Table 5.4 to 5.9) using the following equation:

$d = 1 - N_{XY} / N$

where according to Nei's (1972) formula N_{XY} is the number of bands shared by the individuals Xand Y, and N is the number of total polymorphic sites. The proportion of polymorphic bands (P) was estimated for each population as the number of polymorphic bands divided by the total number of markers analysed.

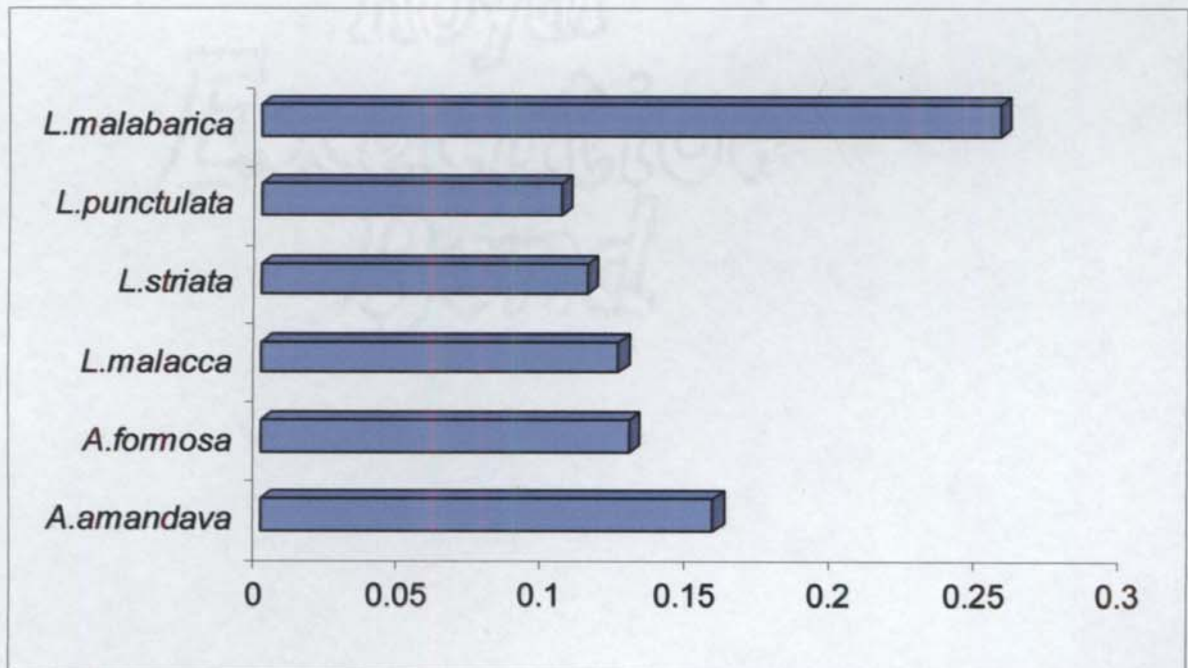
By means of the the Gene Tools software that follows the UPGMA procedure a genealogic dendrogram was constructed, based on values for intra specific distances within individuals of each species.(Figures 5.9 to 5.14).

The genetic distances between individuals of each species are depicted in the dendrograms. The genetic distance values between individuals clearly denotes genetic variation within each species. The mean genetic distance values among individuals of the six species are indicated in the Table 5.3.The graphical representation of the genetic variations observed among individuals of the six species clearly denotes the genetic diversity observed within each species. Among Lonchura, *Lonchura malabarica* or the Indian silver bill exhibits the maximum diversity. It can be assumed as the representative species, from which the populations of L.*malacca, L.striata* and *L.malabarica* may have emerged. Among Estrildae, *A. amandava* is more genetically diverse than *A.formosa.*

Table.5.3 : Mean genetic distance between individuals of each species.

Species	*A.amandava*	*A.formosa*	*L.malacca*	*L.striata*	*L.punctulata*	*L.malabarica*
Mean genetic distance	0.157	0.128	0.124	0.113	0.104	0.256

Figure.5.8: The intra specific variation observed in each species of munia as obtained by the mean genetic distance values among individuals of each species.



598.8860438 TH/R
NB 4958

Table.5.4 Matching coefficients between individuals of ***A.amandava.***

	Track 1	Track 2	Track 3	Track 4	Track 5	Track 6
Track 1	1.000	0.762	0.727	0.727	0.778	0.727
Track 2	0.762	1.000	0.522	0.609	0.526	0.609
Track 3	0.727	0.522	1.000	0.583	0.600	0.583
Track 4	0.727	0.609	0.583	1.000	0.500	0.667
Track 5	0.778	0.526	0.600	0.500	1.000	0.500
Track 6	0.727	0.609	0.583	0.667	0.500	1.000

Figure.5.9 Genealogic dendrogram depicting the genetic distances between the individuals of *A.amandava..* Track 1 to Track 6 indicate six individuals within the species.

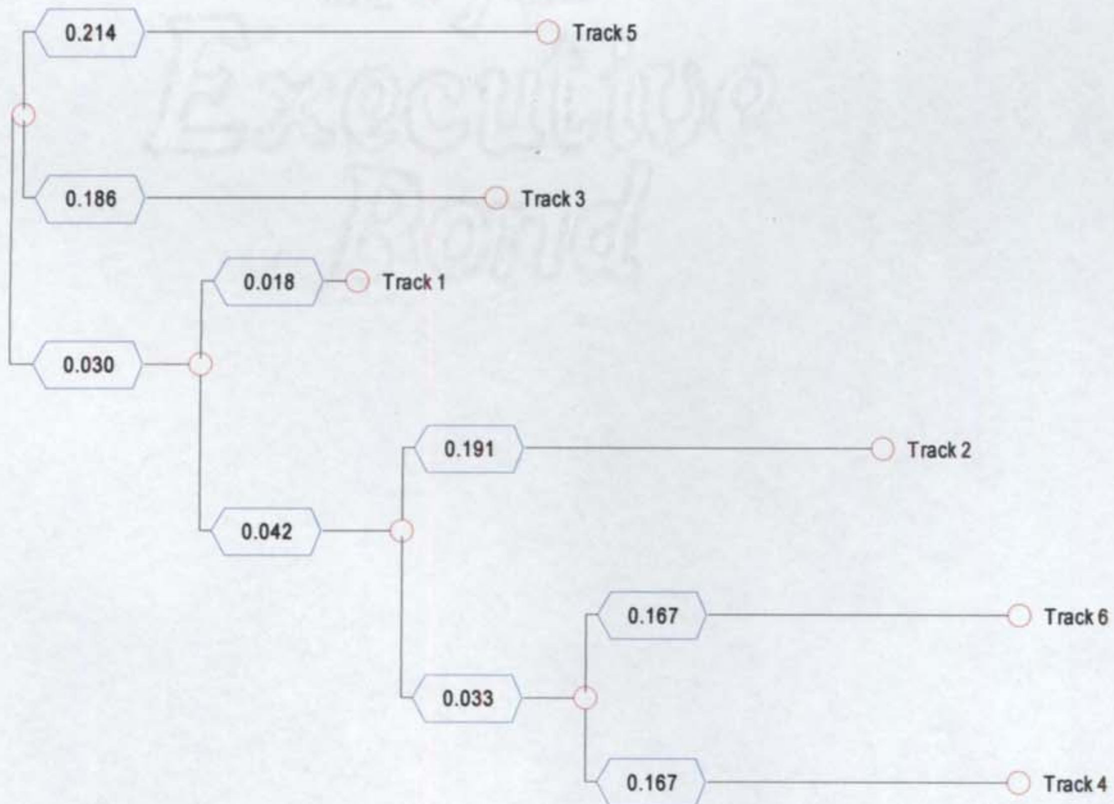


Table.5.5 : Matching coefficients between individuals of ***A. formosa.***

	Track 1	Track 2	Track 3	Track 4	Track 5	Track 6
Track 1	1.000	0.762	0.727	0.727	0.778	0.727
Track 2	0.762	1.000	0.522	0.609	0.526	0.609
Track 3	0.727	0.522	1.000	0.583	0.600	0.583
Track 4	0.727	0.609	0.583	1.000	0.500	0.667
Track 5	0.778	0.526	0.600	0.500	1.000	0.500
Track 6	0.727	0.609	0.583	0.667	0.500	1.000

Figure.5.10: Genealogic dendrogram depicting the genetic distances between the individuals of *A.formosa.* Track 1 to Track 6 indicate six individuals within the species

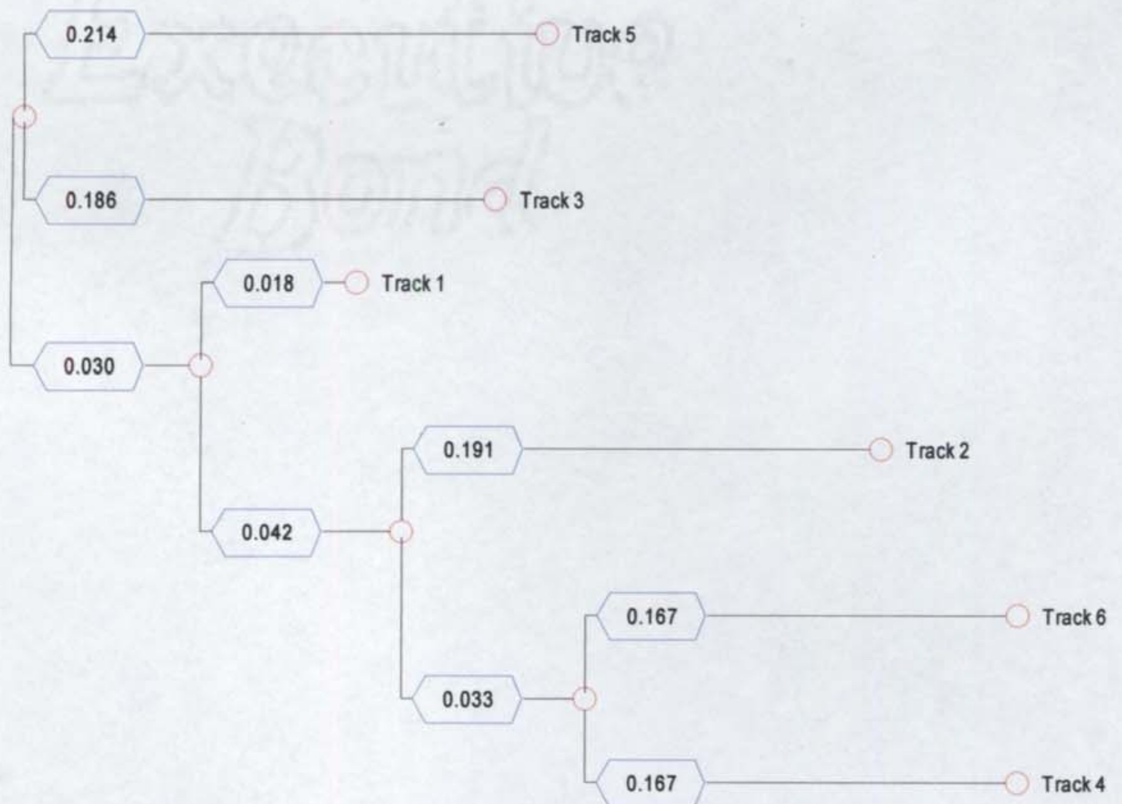


Table.5.6: Matching coefficients between individuals of ***L. malacca.***

	Track 1	Track 2	Track 3	Track 4	Track 5	Track 6
Track 1	1.000	0.923	0.769	0.800	0.800	0.857
Track 2	0.923	1.000	0.714	0.750	0.750	0.800
Track 3	0.769	0.714	1.000	0.625	0.625	0.667
Track 4	0.800	0.750	0.625	1.000	0.667	0.706
Track 5	0.800	0.750	0.625	0.667	1.000	0.706
Track 6	0.857	0.800	0.667	0.706	0.706	1.000

Figure.5.11: Genealogic dendrogram depicting the genetic distances between the individuals of *L.malacca.* Track 1 to Track 6 indicate six individuals within the species.

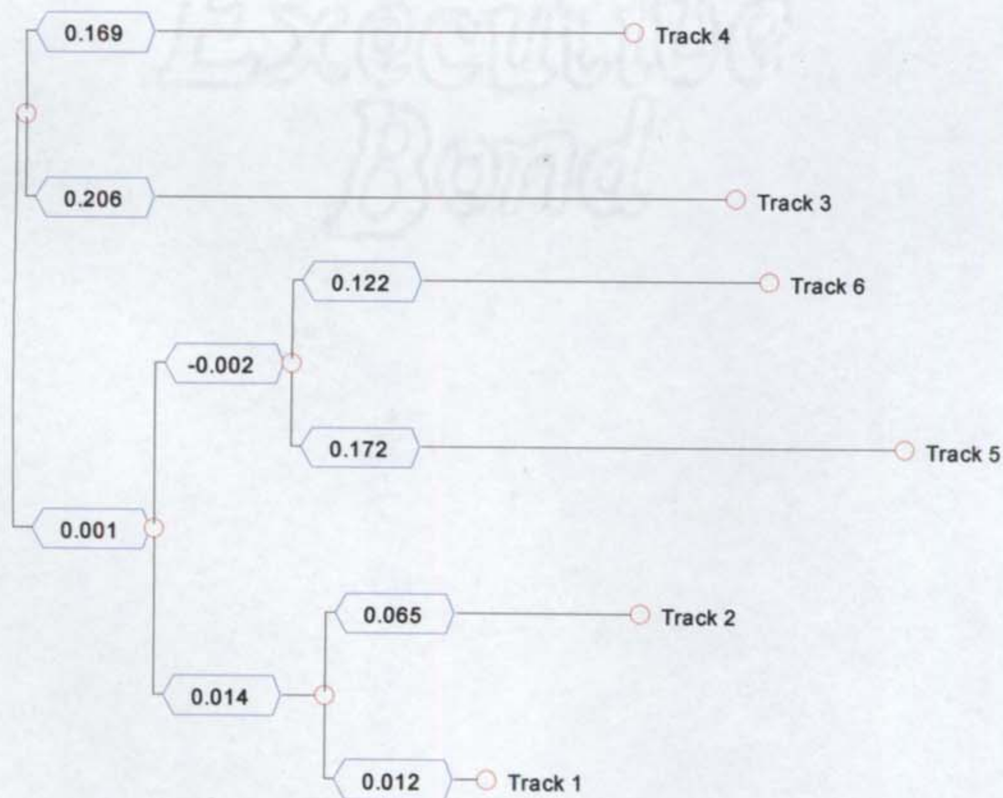


Table.5.7: Matching coefficients between individuals of ***L.striata.***

	Track 1	Track 2	Track 3	Track 4	Track 5	Track 6
Track 1	1.000	0.737	0.889	0.778	0.800	0.889
Track 2	0.737	1.000	0.737	0.632	0.667	0.737
Track 3	0.889	0.737	1.000	0.778	0.800	0.889
Track 4	0.778	0.632	0.778	1.000	0.700	0.778
Track 5	0.800	0.667	0.800	0.700	1.000	0.800
Track 6	0.889	0.737	0.889	0.778	0.800	1.000

Figure.5.12: Genealogic dendrogram depicting the genetic distances between the individuals of *L.striata.* Track 1 to Track 6 indicate six individuals within the species.

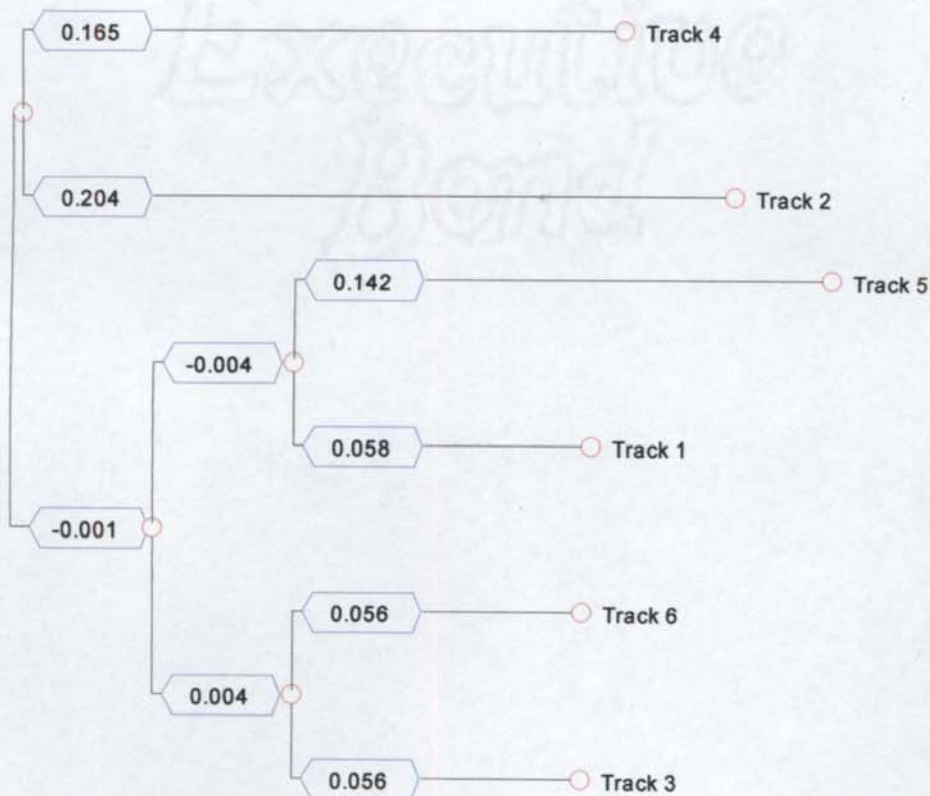
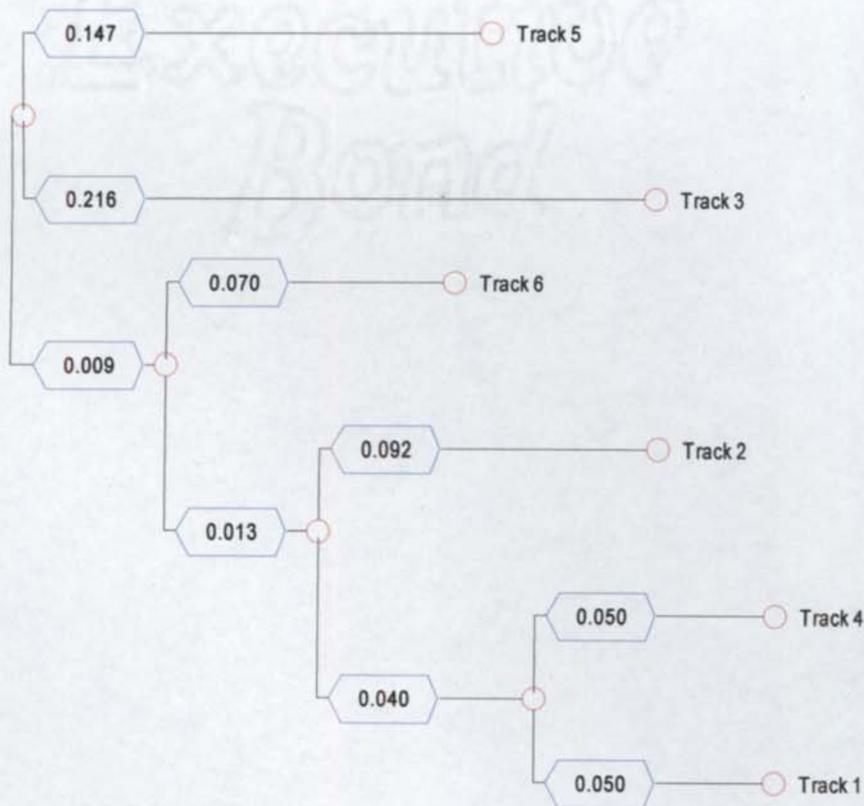


Table.5.8: Matching coefficients between individuals of ***L.punctulata.***

	Track 1	Track 2	Track 3	Track 4	Track 5	Track6
Track 1	1.000	0.818	0.667	0.900	0.762	0.818
Track 2	0.818	1.000	0.696	0.818	0.696	0.833
Track 3	0.667	0.696	1.000	0.667	0.636	0.696
Track 4	0.900	0.818	0.667	1.000	0.762	0.818
Track 5	0.762	0.696	0.636	0.762	1.000	0.783
Track 6	0.818	0.833	0.696	0.818	0.783	1.000

Figure.5.13: Genealogic dendrogram depicting the genetic distances between the individuals of *L.punctulata.* Track 1 to Track 6 indicate six individuals within the species.



5.3: GENETIC DIFFERENTIATION BETWEEN SPECIES.

Genetic variation was observed in all the six species. The species with the highest genetic variation was *L.malacca* with 27% of the bands exhibiting polymorphism. Interestingly no unique bands were detected in *A.formosa*, while three unique bands were detected in *L.striata,* two in L.malacca and one each in *L.punctula*, *L.malabarica* and *A. amandava*. The unique band in Aamandava was considerably prominent and could be sequenced for identification of any unique genetic characterization.

Table.5.10 Banding pattern obtained in the RAPD analysis of each Munia Species

No. Species	Scored	Unique	Polymorphic	Monomorphic
1. *A .amandava*	31	1	9	10
2. *A .formosa*	27	0	5	14
3. *L. malacca*	35	2	12	12
4. *L .striata*	31	3	7	10
5. *L .punctulata*	31	1	5	10
6. *L. malabarica*	32	1	5	11

Table.5.9: Matching coefficients between individuals of ***L.malabarica.***

	Track 1	Track 2	Track 3	Track 4	Track 5	Track 6
Track 1	1.000	0.533	0.667	0.533	0.154	0.706
Track 2	0.533	1.000	0.364	0.714	0.167	0.625
Track 3	0.667	0.364	1.000	0.364	0.000	0.615
Track 4	0.533	0.714	0.364	1.000	0.333	0.750
Track 5	0.154	0.167	0.000	0.333	1.000	0.286
Track 6	0.706	0.625	0.615	0.750	0.286	1.000

Figure.5.14: Genealogic dendrogram depicting the genetic distances between the individuals of *L.malabarica.* Track 1 to Track 6 indicate six individuals within the species.

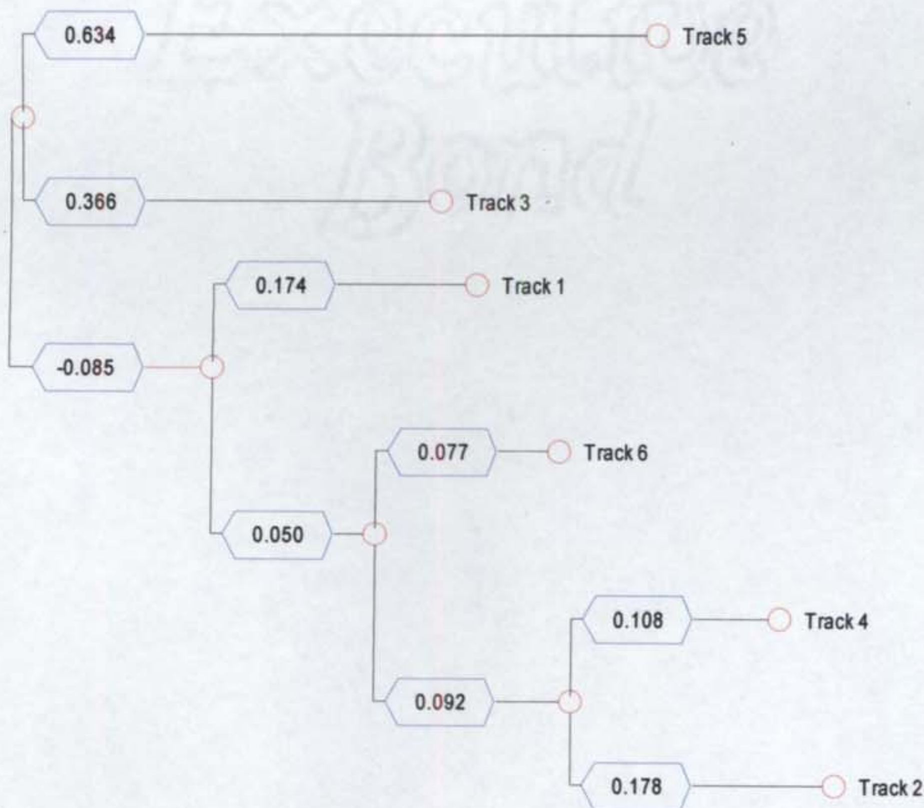
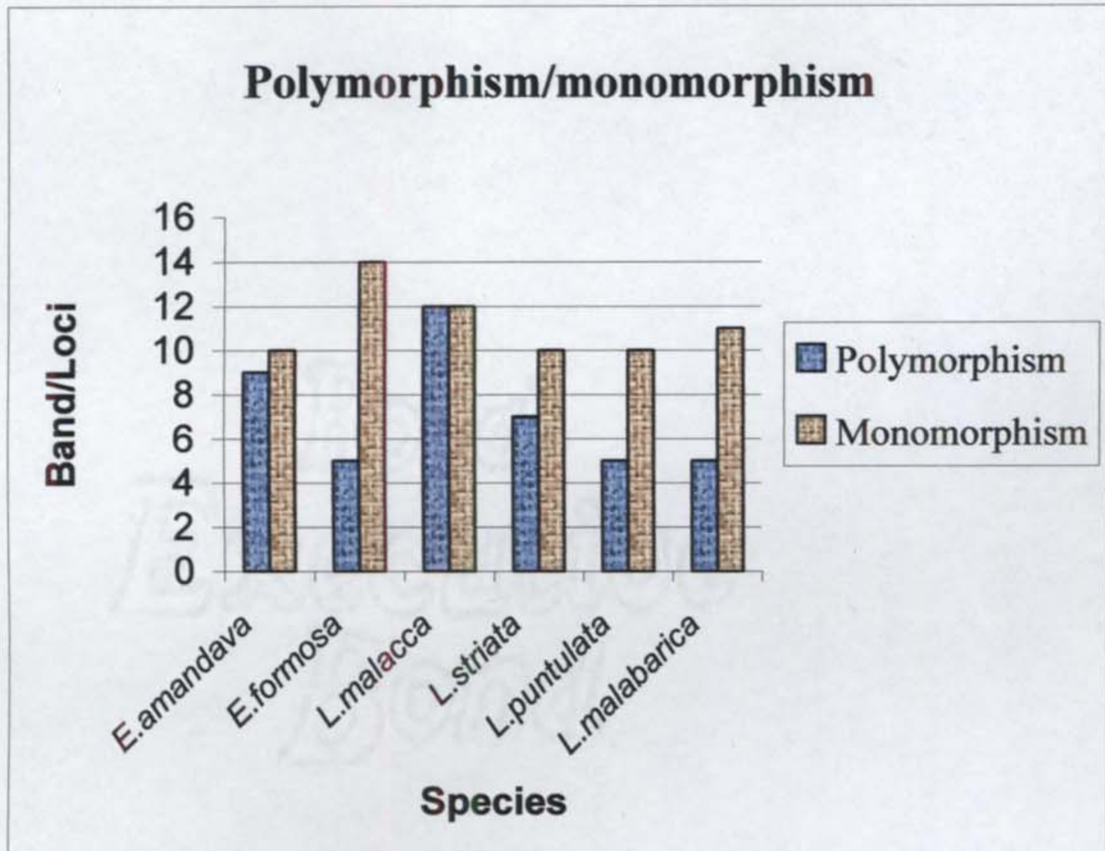


Figure.5.15. Graphical representation of the number of Polymorphic and monomorphic bands obtained in the analysis of intraspecific variance of the six munia species



The six Estrildidae species populations showed that on an average 35% of their RAPD markers were monomorphic with the *Amandava formosa* showing the highest monomorphism. The percentage of monomorphism did not show much range in variability among the other species.The ratio of polymorphic to monmorphic banding was also considerably higher among the *A.Formosa..*

Figure.5.16 Graphical representation of the percentage of Polymorphic bands obtained in the analysis of intraspecific variance of the six munia species

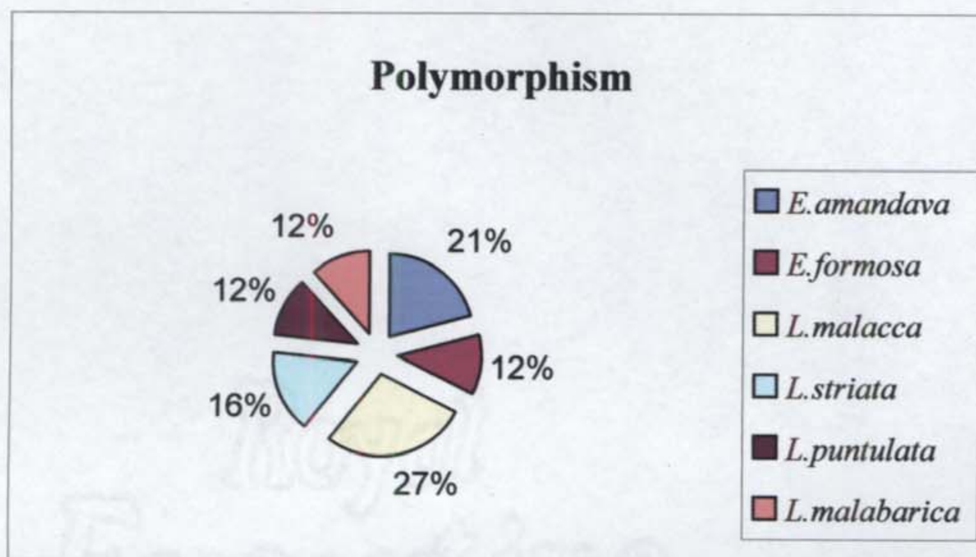


Figure.5.17. Graphical representation of the percentage of Monomorphic bands obtained in the analysis of intraspecific variance of the six munia species

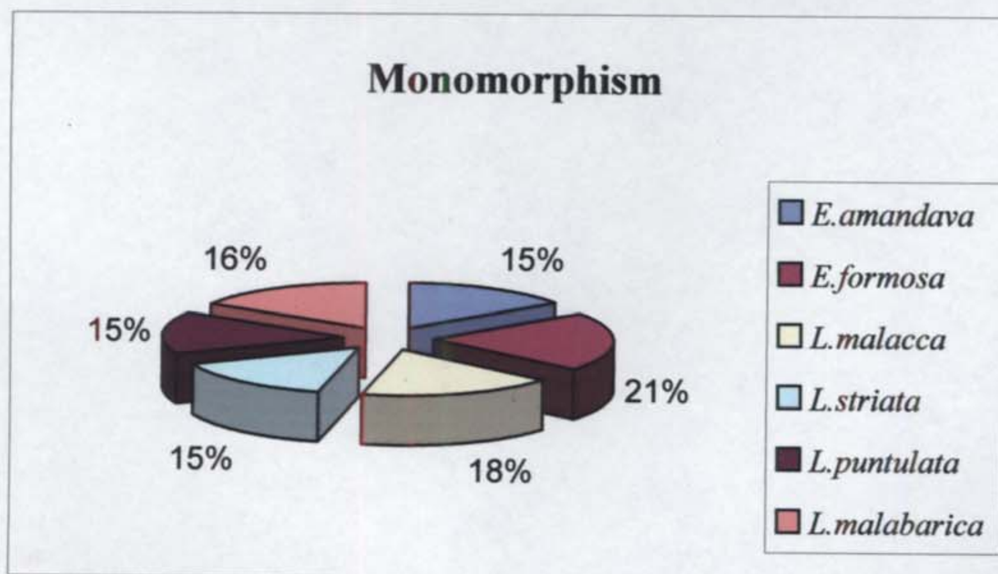


Table.5.11. The proportion of polymorphic banding obtained in the six species of munia.

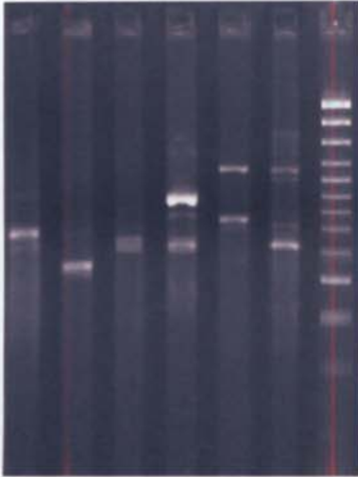
No.	Species	Proportion of polymorphic banding
1.	*E.amandava*	0.048
2.	*E.formosa*	0.026
3.	*L.malacca*	0.053
4.	*L.striata*	0.037
5.	*L.punctulata*	0.043
6.	*L.malabarica*	0.026

TABLE:5.12. Banding Pattern of the 11- RAPD Primers that Amplified 61 Polymorphic bands in pooled DNAs of the six Munia species

Primers	P.bands	Red	Green	Black hd.	White rp.	Spotted	W. throat
p - 1	1	0	0	0	1	1	1
	2	0	0	0	0	1	1
	3	0	1	0	0	0	1
	4	1	1	0	0	0	1
	5	0	0	0	1	1	1
	6	0	0	0	0	0	1
p - 2	7	1	0	0	0	0	1
	8	1	0	0	1	1	0
	9	1	0	1	0	1	0
	10	1	1	1	0	0	0
	11	1	0	1	0	0	0
p - 3	12	1	0	0	0	0	0
	13	1	1	1	0	0	0
	14	1	1	1	0	0	0
	15	1	1	0	0	1	0
	16	1	0	0	1	0	0
	17	1	0	1	1	0	0
	18	1	1	1	0	0	0
p - 4	19	1	0	0	1	0	1
	20	1	0	0	1	0	1
	21	0	1	1	0	0	1
p - 5	22	0	1	0	0	0	1
	23	0	0	0	0	0	1
	24	0	0	1	0	0	1
	25	0	0	0	0	1	1
	26	0	0	0	1	1	1
	27	1	0	0	1	0	1
	28	1	0	0	0	0	1
	29	0	0	1	0	0	1
	30	0	0	0	0	0	1
	31	1	0	1	0	0	1

Primers	P.bands	Red	Green	Black hd.	White rp.	Spotted	W. throat
p - 6	32	0	0	0	0	0	1
	33	0	0	0	1	0	1
	34	0	0	1	1	0	1
	35	0	0	0	0	0	1
	36	1	0	1	0	0	1
	37	1	1	0	0	0	1
	38	0	1	0	0	1	1
	39	1	0	0	0	0	1
p - 7	40	1	1	0	0	0	1
	41	1	0	0	0	0	0
	42	1	0	0	1	0	0
	43	1	0	0	1	0	1
p - 8	44	0	1	0	1	0	1
	45	0	0	1	0	1	1
	46	0	0	0	0	0	1
p - 9	47	0	0	0	0	0	1
	48	1	1	0	0	0	1
	49	1	0	0	0	1	1
	50	1	0	0	0	0	1
p - 10	51	0	0	0	0	1	1
	52	0	0	0	0	1	1
	53	0	0	0	1	1	1
	54	0	0	0	0	0	1
	55	0	0	0	1	1	1
	56	0	0	0	1	0	1
	57	1	0	0	0	0	1
p - 11	58	0	0	0	0	1	1
	59	0	0	1	0	0	1
	60	1	1	0	0	0	1
	61	0	1	0	0	1	1

Plate 5.8: RAPD banding between the six Estrildidae species with primer Nos. 1& 2 and images of their band sharing



PRIMER –1

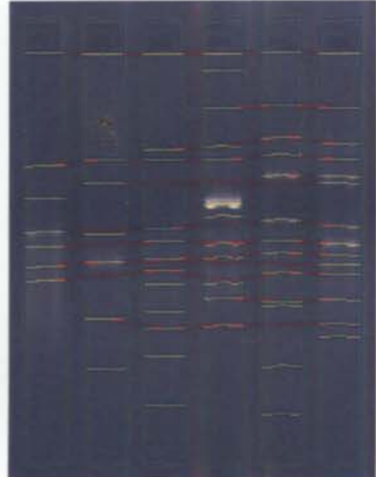
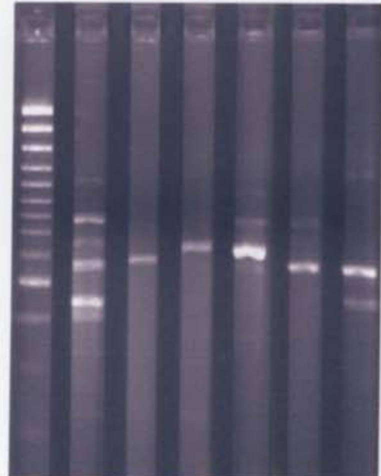


IMAGE – 1

IMAGE - 2



PRIMER - 2

Plate 5.9: RAPD banding between the six Estrildidae species with primer Nos. 3& 4 and images of their band sharing

PRIMER – 3

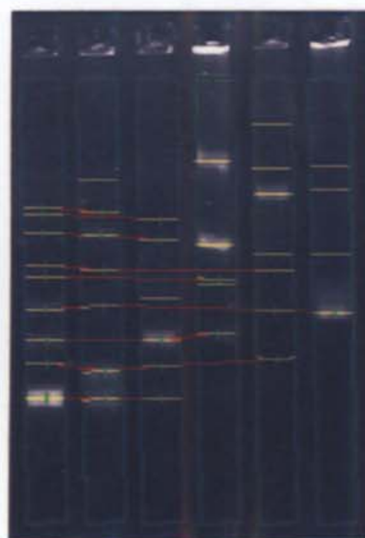
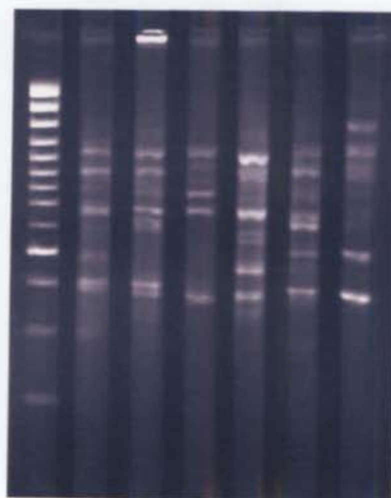


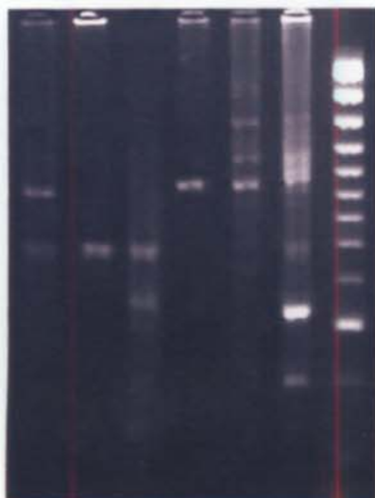
IMAGE - 3

IMAGE—4



PRIMER - 4

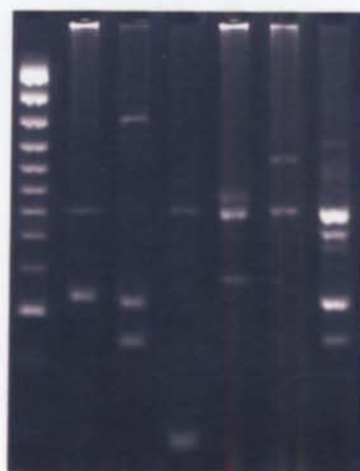
Plate 5.10: RAPD banding between the six Estrildidae species with primer Nos. 5 & 6 and images of their band sharing



PRIMER –5

IMAGE – 5

IMAGE – 6



PRIMER – 6

Plate 5.11: RAPD banding between the six Estrildidae species with primer Nos. 7 & 8 and images of their band sharing

PRIMER – 7

I MAGE - 7

IMAGE – 8

PRIMER - 8

Plate 5.12: RAPD banding between the six Estrildidae species with primer Nos. 9 & 11 and images of their band sharing

RIMER – 9

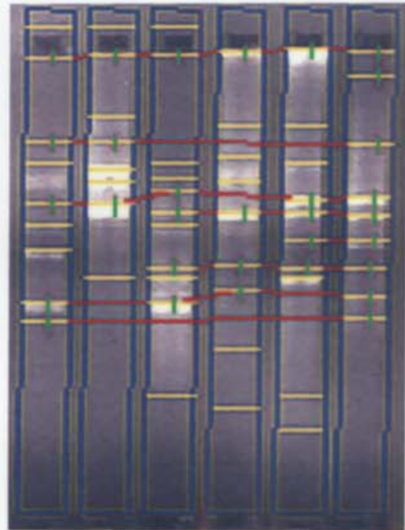
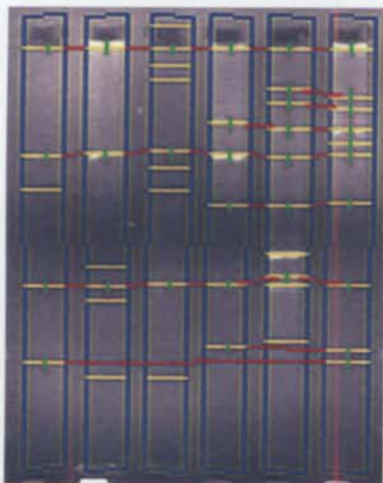


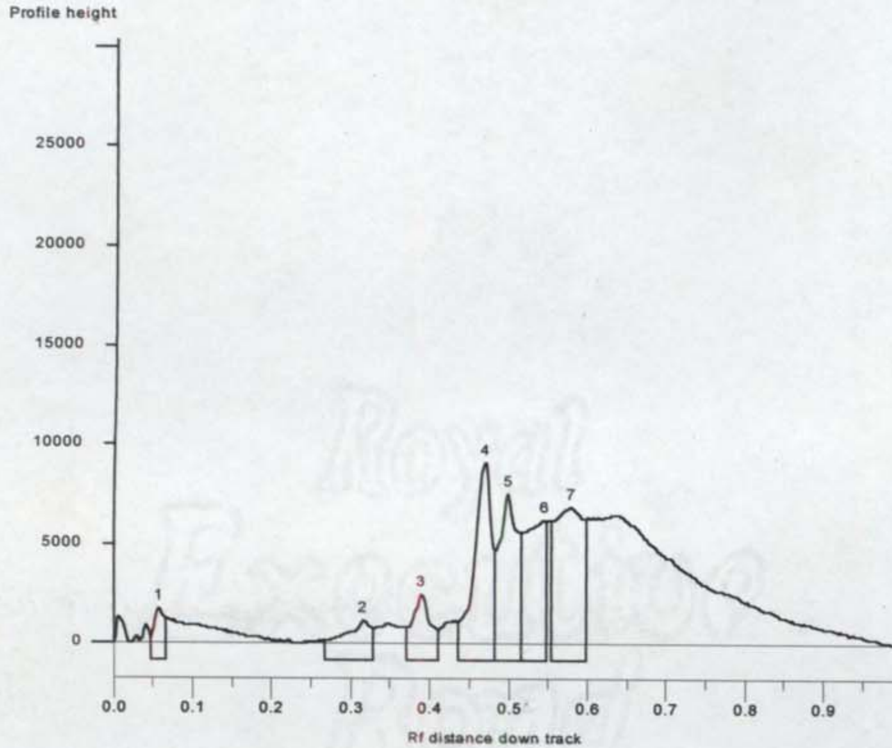
IMAGE - 9



PRIMER – 11

IMAGE – 11

Figure.5.18. Profile analysis of the track that represents the ***E.amandava*** species to identify amplified products of RAPD using primer-1.



Bands	Height
1	1806.8
2	1155.495
3	2533.452
4	9146.982
5	7514.476
6	6224.134
7	6876.609

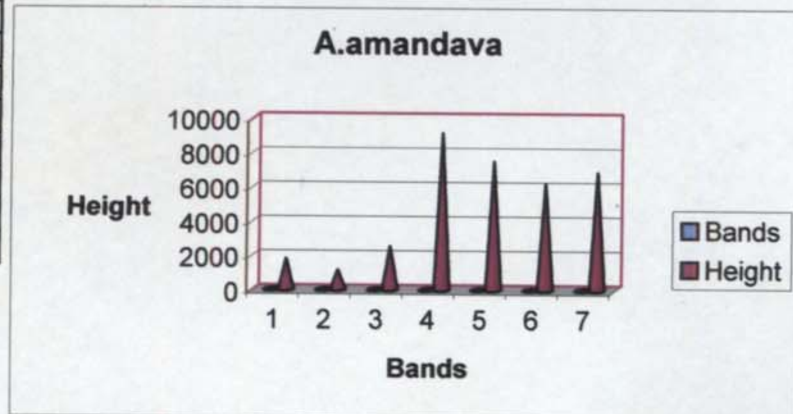
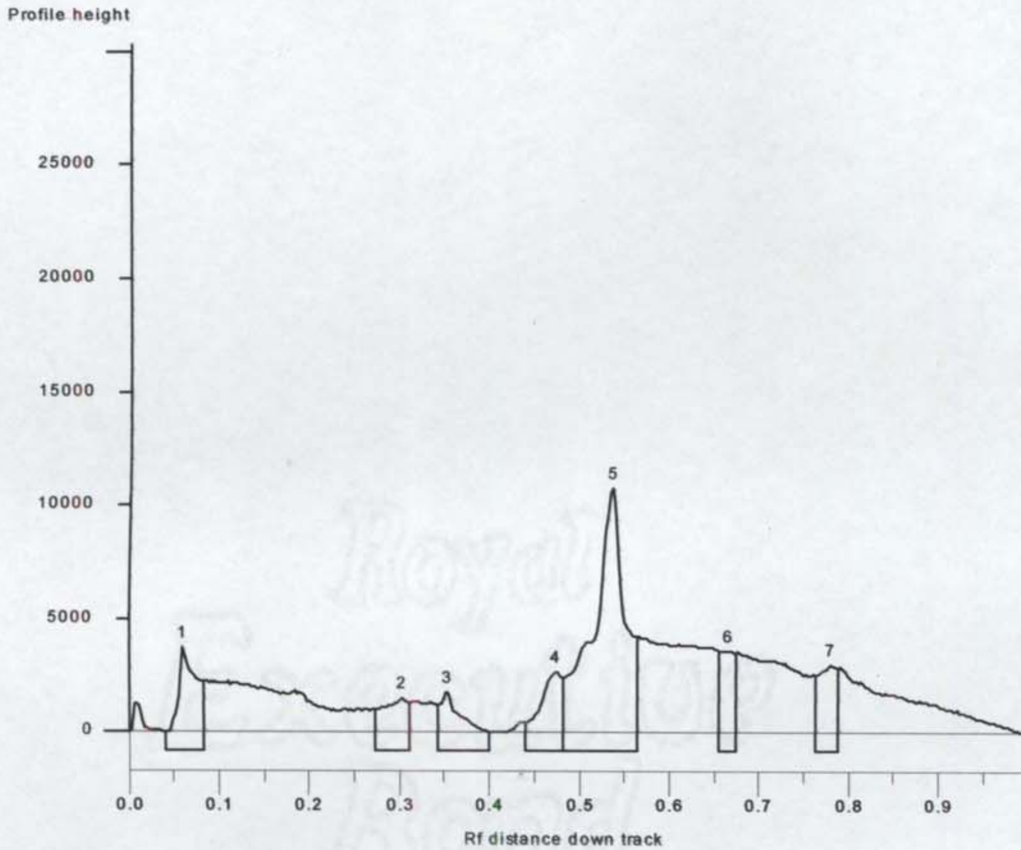


Figure.5.19: Profile analysis of the track that represents the ***E.formosa*** species to identify amplified products of RAPD using primer-1.



Bands	Height
1	3736.312
2	1494.006
3	1782.851
4	2676.053
5	10734.232
6	3624.582
7	3017.37

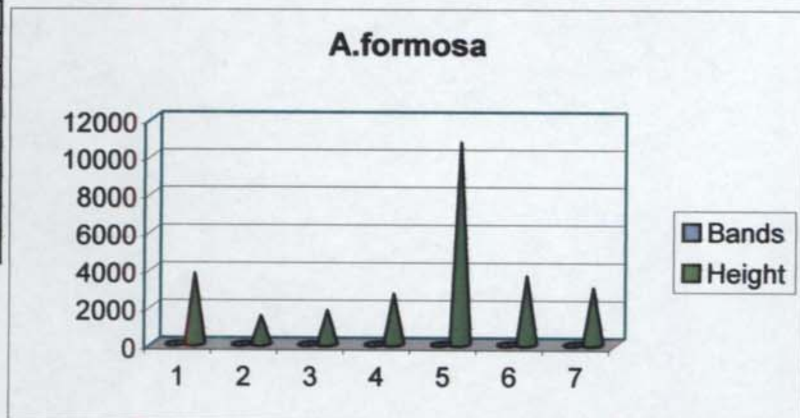
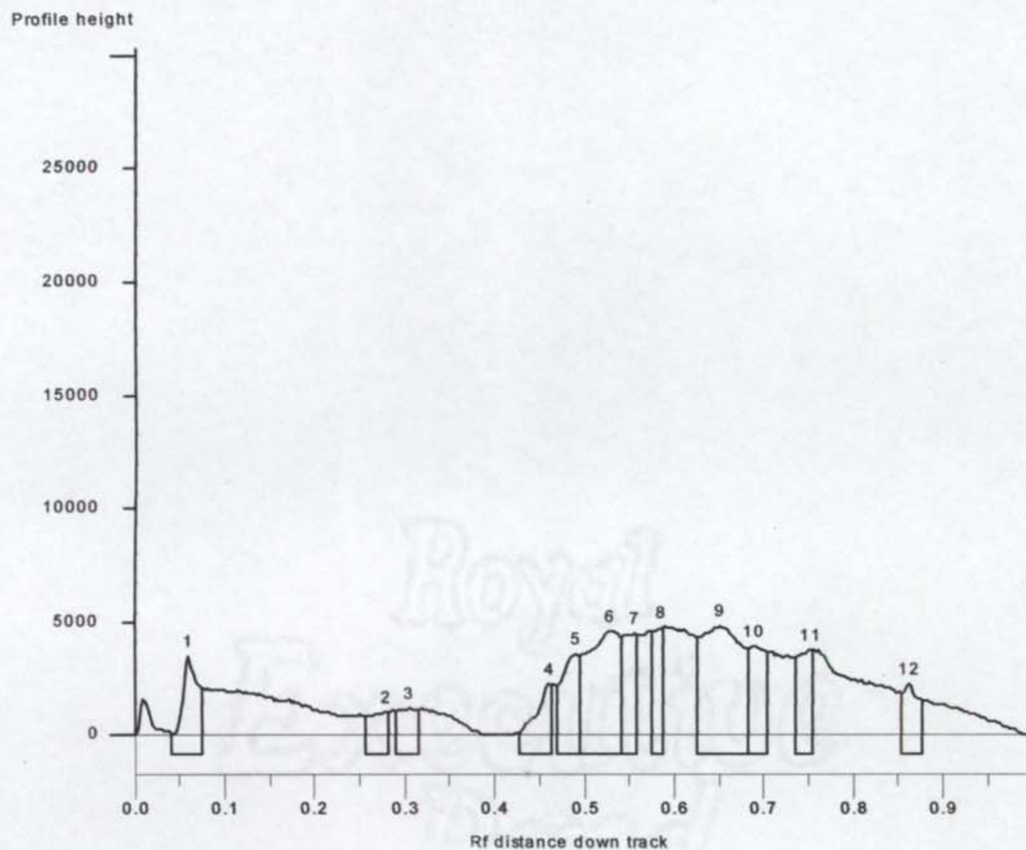


Figure.5.20: Profile analysis of the track that represents the ***L.malacca*** species to identify amplified products of RAPD using primer-1.



Bands	Height
1	3465.268
2	1027.724
3	1191.132
4	2304.034
5	3593.282
6	4619.81
7	4458.334
8	4718.626
9	4805.07
10	3920.949
11	3780.072
12	2244.644

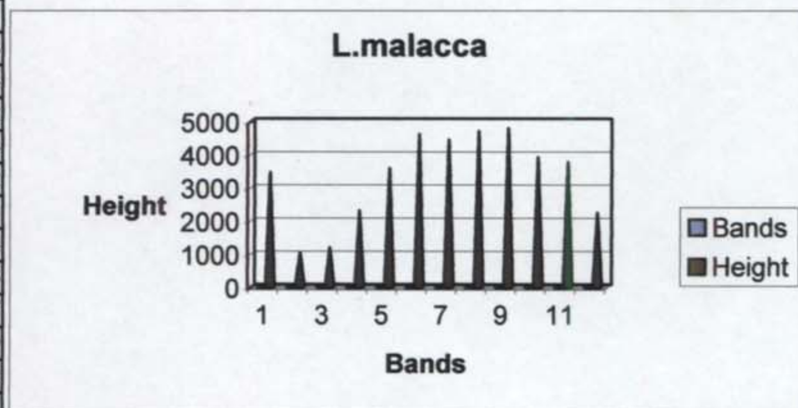
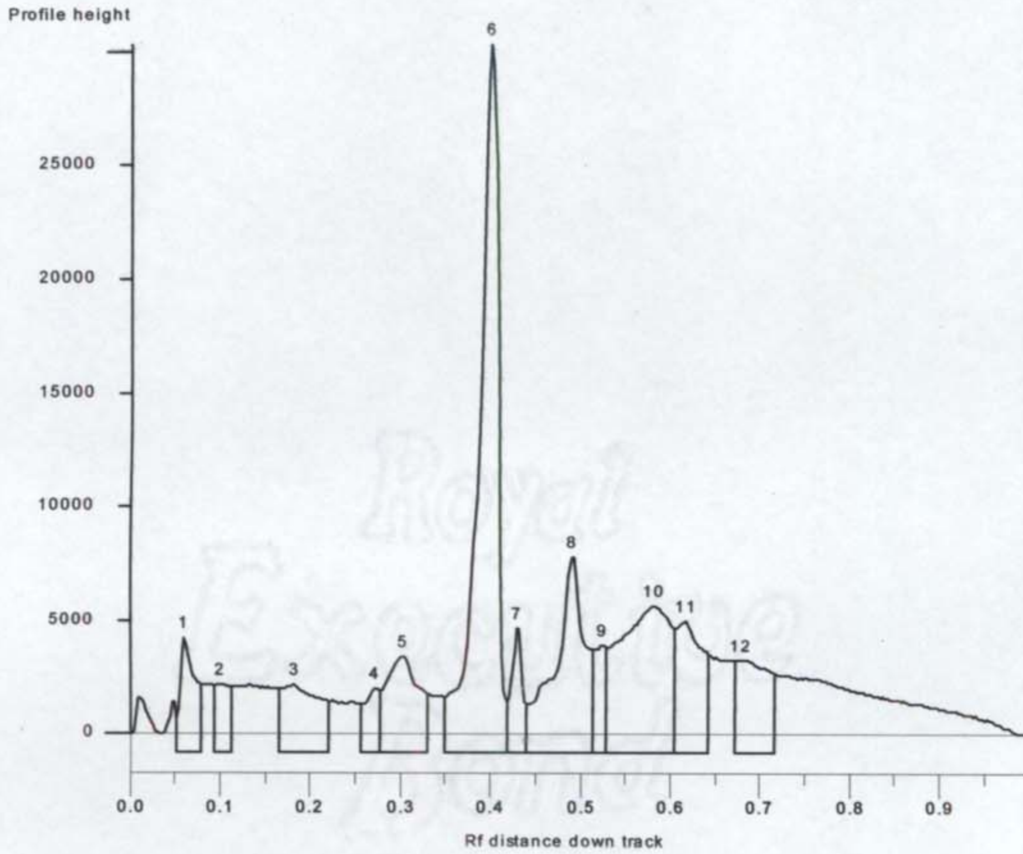


Figure.5.21: Profile analysis of the track that represents the ***L.striata*** species to identify amplified products of RAPD using primer-1 .



Number	Height
1	4255.162
2	2180.966
3	2179.707
4	2012.412
5	3462.664
6	30361.393
7	4684.004
8	7780.829
9	3912.272
10	5677.348
11	4951.987
12	3277.32

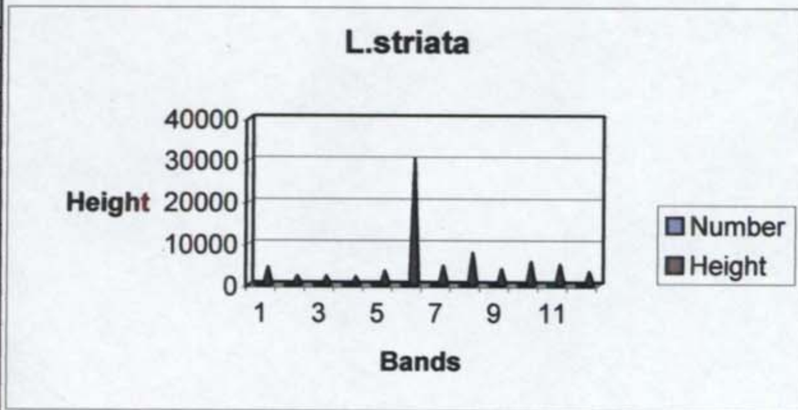
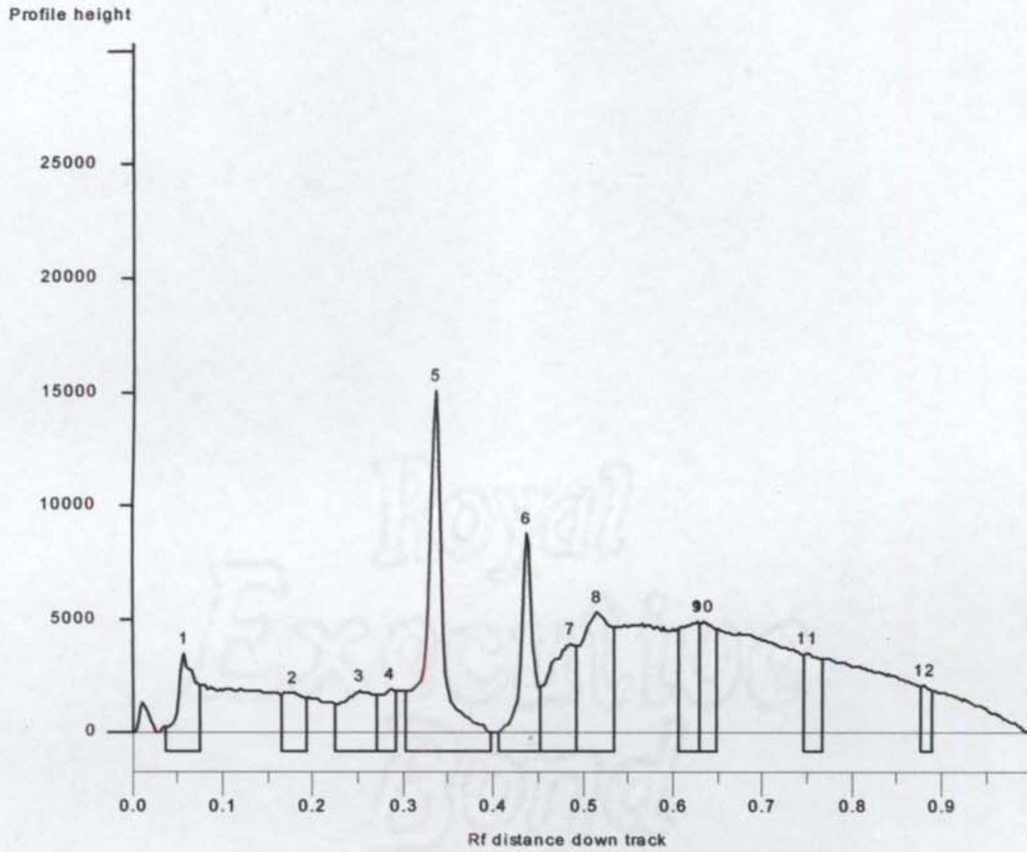


Figure.5.22: Profile analysis of the track that represents the ***L.punctulata*** species to identify amplified products of RAPD using primer-1.



Number	Height
1	3482.286
2	1786.395
3	1836.018
4	1899.746
5	15082.228
6	8766.75
7	3903.124
8	5284.627
9	4884.728
10	4867.915
11	3509.532
12	2105.71

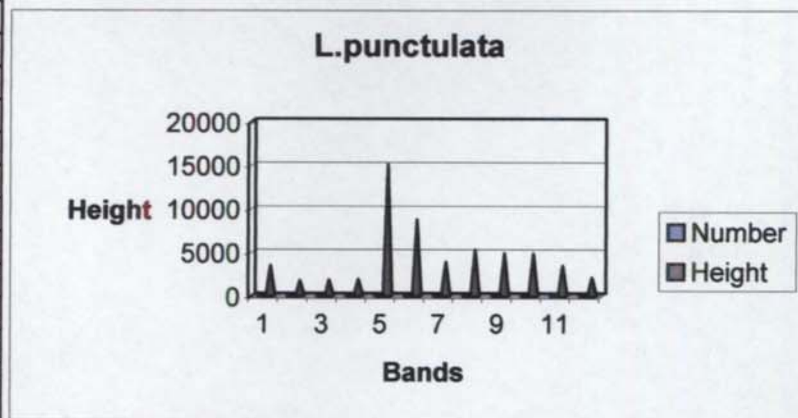
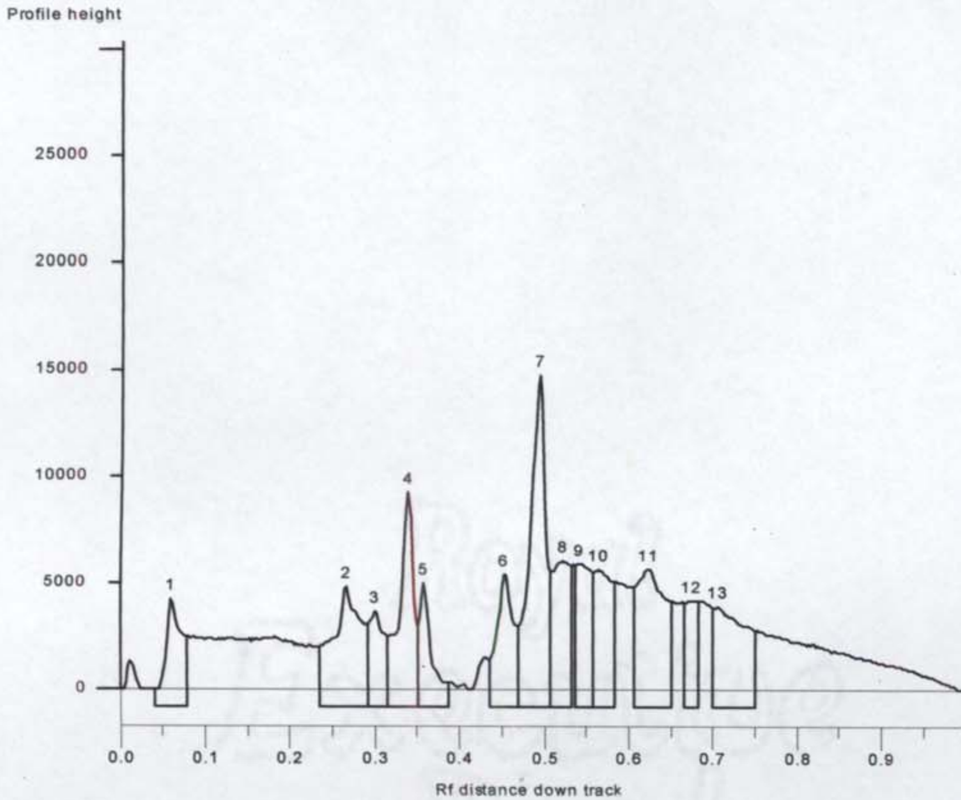


Figure.5.23: Profile analysis of the track that represents the ***L.malabarica*** species to identify amplified products of RAPD using primer-1.



Number	Height
1	4229.504
2	4840.467
3	3678.698
4	9260.638
5	4966.916
6	5412.778
7	14753.489
8	6060.59
9	5930.155
10	5631.84
11	5666.118
12	4182.548
13	3903.23

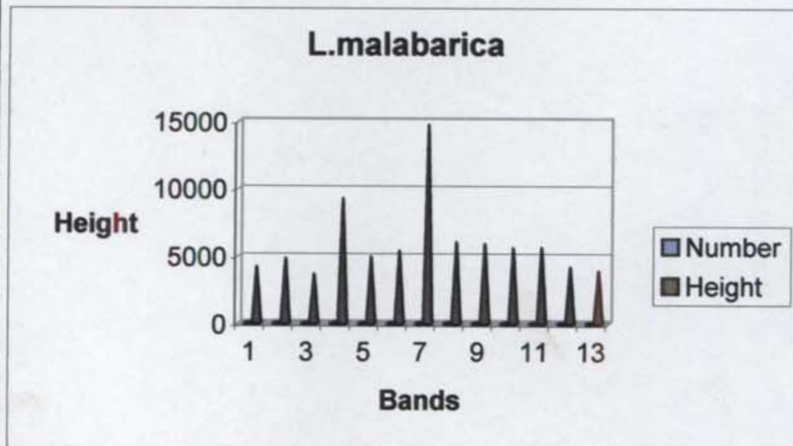


TABLE:5.13. Matching presence/absence---PRIMER - 1 (AGCGTCCTCC)

Matching reference track is on the gel: Track 6 – Based on Position

Peak	Track 1	Track 2	Track 3	Track 4	Track 5	Track 6
1		X	X	X	X	X
2				X	X	X
3			X	X	X	X
4	X	X	X	X	X	X
5					X	X
6		X				X
7	X		X		X	X
8	X		X	X	X	X
9			X	X	X	X
10	X	X				X
11	X		X	X		X
12				X	X	X
13		X		X		X
14			X			X
15						X

TABLE: 5.14. Matching coefficients between the six species of ***Estrildidae***. Each track represents each species.

	Track 1	Track 2	Track 3	Track 4	Track 5	Track 6
Track 1	1.000	0.429	0.526	0.421	0.500	0.571
Track 2	0.429	1.000	0.316	0.316	0.200	0.476
Track 3	0.526	0.316	1.000	0.583	0.560	0.615
Track 4	0.421	0.316	0.583	1.000	0.640	0.692
Track 5	0.500	0.200	0.560	0.640	1.000	0.741
Track 6	0.571	0.476	0.615	0.692	0.741	1.000

5.4: GENETIC RELATIONSHIPS WITHIN *ESTRILDIDAE*

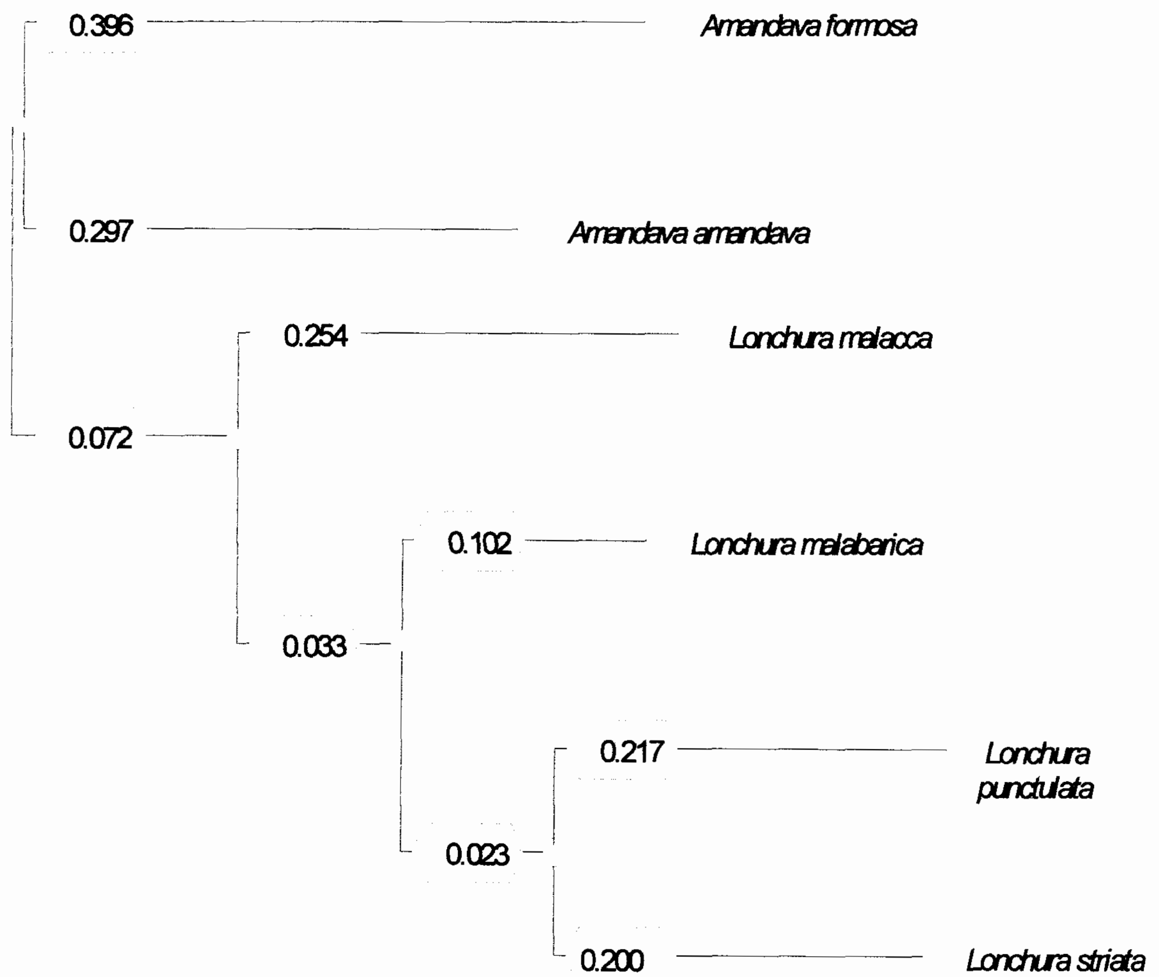
By means of the UPGMA procedure, a genealogic dendrogram was constructed, based on values for interspecific distances within the Estrildidae. This dendrogram reveals that the family is divided into two distinct genera, separated by a genetic distance of 0.441. One group fits well with the currently recognized waxbills (Estrildinae) and the other comprises the so called mannikins (Lonchurae)The present classification of the family into genera Lonchurae and Estrildinae by Mayer et al 1968 is reconfirmed by the genetic analysis.

At the species level, the mean genetic distance was 0.153 which was slightly higher than the avian mean (Avise et al. 1982). The mean genetic distance between genera within the Estrildidae was found to be 0.346.

Classification of Estrildidae based on phenotypic variations of protein electrophoretic patterns need not be reconsidered in the context of molecular analyses by RAPD. Kakizawa and Watada studied 42 species belonging to 17 genera among the Estrildidae and divided them into two main groups, the Estrildinae and the Lonchurinae. The Esrildinae is composed of the three groups, the Estrildi, Erythruri, and Amadini, while the Lonchurinae consist of the Poephili, Lonchuri and Heteromunii. The basic taxonomic arrangement of genera and species agreed with the present findings. These results also support the hypothetical African origin of the Estrildidae with later dispersal to Asia and Australia. Thus, RAPD based molecular markers were able to identify the differences between the species which were revealed by electrophoretic protein based markers.

However, additional phylogenetic studies using chloroplast or mitochondrial gene sequences or appropriate nuclear gene sequences can help to evaluate the systematic positions of these species.

Figure: 5.24. The Genealogic dendrogram based on UPGMA of Matching Coefficient derived from the band matching reference table; depicting the genetic distances between the six species of ***Estrildidae.***



RECONSTRUCTION OF PHYLOGENY OF BIRDS OF THE GROUP ESTRILDIDAE BY USING DNA COMPARISONS AND DNA MARKERS

THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSPHY IN ZOOLOGY

by

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OCTOBER – 2004

CHAPTER VI

DISCUSSION

6.1 GENETIC VARIATION AND DIFFERENTIATION IN ESTRILDIDAE

6.2 PHYLOGENETIC RELATIONSHIPS WITHIN ESTRILDIDAE

6.3 MOLECULAR GENETICS AND CONSERVATION

6.4 CONCLUSION

6. DISCUSSION

During the past few years, there has been considerable debate concerning the possible effects of fragmentation, isolation and small population size on the genetic variability of bird populations. The high vagility of many bird species typically results in high levels of gene flow and thus little local genetic differentiation. (Barrowclough, 1980). Only a limited number of studies have demonstrated genetic effects as a result of fragmentation of continental bird populations. (Stangel etal. 1992; Haig et al 1993).This study evaluated the population genetic structure of the six species of Estrildidae found in the subcontinent.

The present study reveals that PCR based fingerprinting technique, RAPD is an informative methodology for estimating the extent of genetic diversity as well as to determine the pattern of genetic relationships between different species of *Estrildidae*, with polymorphism levels sufficient to establish informative fingerprints with relatively fewer primer sets. The RAPD procedures proved to be a useful tool for assessing genetic variability because band profiles with the selected primers were reproducible. Despite the high proportion of excluded bands a substantial number of polymorphic markers was detected. The information obtained from the present study could be of practical use for mapping the *Esrildid*ae genome.

6.1: GENETIC VARIATION AND DIFFERENTIATION OF ESTRILDIDAE

This study of the Estrildidae found that the observed values of the proportion of polymorphic markers were very similar to, although slightly lower than the overall values for the Passeriformes (Kakizawa and watada,1985) . Although the species studied are popularly caged, most of the material came from wild populations, thus the lower value cannot be

considered to be attributable to random genetic drift due to artificial breeding, but rather to a peculiarity of the Estrildidae.

Within Estrildidae, the mean genetic distance at the species level was calculated as 0.243 which are slightly higher than the avian mean of 0.123 (Avise et al, 1982). This difference can be attributed to the high values exhibited by the two species of Amandava 0.396 and 0.297 by *A. formosa* and *A. amandava* respectively.

In the study the two species of Estrilda, *Amandava amandava* and *Amandava formosa* recognized by Kakizawa and Watada (1985) as belonging to the subgroup Estrildi of the group Estrildinae have been analyzed for their population genetic structure to identify their relationships and variations. The genus Estrilda represent a group that has separated relatively early. Owing to the plumage coloration they are distinct from the mannikins and are commoly called the waxbills. Inspite of their relationships within the genus , their genetic distance values (0.396 and 0.297)reveal that they are distantly related. Wolters (1975 –85) had placed these two species in different genera owing to the aberrations in their characters. But the mean within species genetic distance values of the two species indicate that *A.formosa.* is less genetically diverse than *A.amandava.* *A. formosa* is an endemic species of central India. But the within population genetic variation measured by percentage band sharing and percentage polymorphism reveal that variations in *A. formosa* are not significantly low compared to the other munia species. Inspite of that they are categorized as vulnerable, owing to their deteriorating habitat and endemism.

The group Lonchurinae, commonly called mannikins show no sexual dimorphism. They form a uniform group in a single genus the Lonchura.They are widely distributed in Africa , South-East Asia, India and Australia. The four species considered; *L. malabarica, L. malacca, L. punctulata* and *L .striata* are the common species in India.(Manakadan and

Pittie, 2001) They show low levels of genetic distances within them indicating a relatively recent genetic differentiation of the group.

By the analysis of genetic distances between individuals of each species the amount of within species genetic diversity was estimated. The mean genetic distance values indicated that all the four species exhibit considerable diversity with them. Among the species; *L.punctulata* and *L.striata* with the shortest mean genetic distances (0.104 and 0.113) can be considered the closest relatives and the most recently diverged species.*L. malacca* with a mean genetic distance value of 0.124, shows a greater diversity. *L. malabarica* commonly called the Indian silver bill or the White-rumped munia shows a significantly high genetic diversity(0.256). It is a well established species of the subcontinent (Harrison, 1964). The values clearly indicate that the *L. malabarica* is the true representative of the Lonchura population of the subcontinent and that populations of *L. punctulata. L.striata,* and *L.malacca* may have emerged later from it.

6.2: PHYLOGENETIC RELATIONSHIPS WITHIN THE ESTRILDIDAE

The results showed that the mean genetic distance between the species of Estrildidae was 0.346 between the genus Estrilda or Amandava while in the genus Lonchura the value of mean genetic distance was only 0.153. As the mean genetic distances between species in the Estrildinae was higher than that of those in the Lonchurinae, and from the values obtained it is observed that the rate of genetic differentiation decreased from the species Estrildinae to the species Lonchurinae, reflecting a progressively more recent speciation in which the older species are found in the Estrildinae. However, with further studies on more species and more number of loci changes of the interspecific relationships within the Lonchurinae and Estrildinae can be expected.

The Estrildidae are divided into two main groups with a genetic difference of 0.44 (Kakizawa and Watada, 1985). Both of these groups; the Estrildinae and the Lonchurinae each consist of sub groups, named: (1) Amadini (2) Estrildi (3) Erythruri (4) Poephili (5) Lonchuri and (6) Heteromunii (Kakizawa et al 1985).

Of the six species considered for the study, the two Amandava species; *A. amandava* and *A.Formosa* belonged to the sub group Estrildi of the group Estrildinae. Estrildi comprise of many genera which separated relatively early. The species of Estrildi are known to show much variation even within the same genus. They may be closely or sometimes very distantly related.

In this study from the dendrogram it is clear that the group Estrildinae consisting of the two species had separated relatively early. Kakizawa had observed a further division of this group into subgroups of which *A. amandava* is an older species belonging to the older subgroup. From the present study also it can be considered that the species *A. Amandava* had branched off first from an ancestral group common to the Estrildinae group and had remained undifferentiated. Enjoying a wide spread distribution the Red munia can be considered as the representative species which has undergone the least evolutionary pressure.

A..formosa , the Green Munia on the other hand is a species that has separated out later and became endemic to the parts of north India. With a red bill on a green body its attraction as cage bird has made it vulnerable. Present study indicated that the Green Munias had considerable within population variation. Therefore translocations between population to promote gene flow is likely to have little or no influence on maintaining the population levels beyond vulnerability. Thus management interventions to acquire and maintain quality habitat that promote population increases and migration are likely to be the most beneficial measures. The green munia population being genetically unique compared to other species of Estrildidae

in the subcontinent, may be considered as an important evolutionary unit of conservation.

The four species of Lonchura obtained for the study belonged to the subgroup Lonchuri of the group Lonchurinae. They form a uniform group represented by a single genus Lonchura with no sexual dimorphism bright plumage coloration. They are all somber coloured. The Lonchuri consists of three groups (Delacour,1943., Mayer et al. 1968) based on their distribution, the African, Indian and the Australasian. The species of lonchura are very closely related to each other with a genetic distance of less than 0.219. The low levels of genetic distance within Lonchuri indicates the relatively recent genetic differentiation of this group. The four species of Lonchura has shown considerable within species genetic variability. They reflect a continuous and abundant distribution. They are all common pests of agriculture and may therefore be considered for control and management programmes in agriculture.

A fifth species of Lonchura, *L.kelaarti*, the Black throated munia endemic to the Western Ghats though chosen for the study, was not obtained during the course of the work. The search for the bird during the course of the study has bought up many questions, which has triggered the need for future research on the endemic species of Estrildidae, both *A. Formosa* and *L.kelaarti* in India (Gaston,1983). In 1983 Gaston has observed that species characteristic of the heavily disturbed lowland ecosystems of the Indogangetic plain and the peninsular India show signs of increased rate of extinction or vulnerability to extinction. With its preferred habitat of the high range forest vegetation, the species *L.kelaarti* has become highly localized and uncommon. (Satish et al, 2001). The species is distributed in the high rainfall, moist deciduous and evergreen forests of southwest India and the Western Ghats. This forest has been much reduced by commercial timber extraction and the construction of hydropower dams, but areas of seminatural forests remain except in the narrow strip of coastal plains where natural forest has

been practically eliminated. With further reduction of their preferred habitat, *L.kelaarti* could easily become vulnerable to extinction.

6.3: MOLECULAR GENETICS AND CONSERVATION

Conservation biology seeks to maintain both unique species and the genetic diversity within those species. Without the help of molecular genetics , however, it's not necessarily easy to quantify either quality. Using mitochondrial DNA, polymerase chain reactions techniques, and microsatellite DNA, molecular genetists help biologists determine which species are genetically distinct and weather their DNA are adequately diverse, allowing wise management of endangered species.

Effective conservation and restoration plans for species at risk of extinction or extirpation require clearly definable units of management. Currently, most management units are arbitrarily defined as meta populations , which inhabit contiguous and adequate habitat. Classically, the principle processes in meta-population dynamics are extinction, migration, and colonization to establish new local populations. The key questions are how these processes jointly affect the dynamics and the evolution of local populations within which the conspecifics are more likely to interact with other than with conspecifics from other populations. However, isolation is usually not complete and since most organisms have some power of dispersal, members of a local population have a low but positive probability of interaction with individuals from other localities. Depending on the rate of emigration, demographic and genetic dynamics will be influenced by this migration, as well as by local birth and death rates. Genetic information is rarely available on population structures, levels of gene flow, or relatedness of geographic populations of many species of conservation concern. To develop management strategies for maintaining evolutionarily significant lineages that will ensure long – term population stability and reduce the need for protection through the regulatory process, a thorough understanding of the evolutionary

relationships (e.g., levels of gene exchange) among geographically proximate and distal populations are essential. Molecular genetics has recently achieved an important place in contemporary conservation biology as it has proven to be a robust tool for identifying reproductive isolation among populations, permitting the delineation of management units, and allowing assessment of conservation procedures for an evolutionary perceptive.

The recognition of recent accelerated depletion of species as a consequence of human industrial development has spawned a wide interest in identifying threats to endangered species (Gaston, 1980,1984). In addition to ecological and demographic perils it has become clear that small populations that narrowly survive demographic contraction may undergo close inbreeding genetic drift, and loss of overall genomic variation due to a allelic loss of reduction to homozygosity.

6.4: CONCLUSION

The genetic similarity of *Amandava* with *Lonchura* species is low as indicated by the separation of these two groups in the RAPD analyses. The informative primers identified in the studies will be useful in genetic analysis of *Estrildidae* species. The putative species-specific bands can be used as probes to ascertain whether they are in low or high copy numbers in the *Estrildid* genome, and such specific bands may be used for genotype characterization. Further, putative species-specific RAPD markers could be converted to sequence characterized amplification regions (SCARs) after sequencing and designing primer pairs to develop robust species specific markers (Fritsch, 1996). The study also provides a basis for conservationists to make informed choices on selection of parental material based on genetic diversity to help overcome some of the problems usually associated to develop management strategies for maintaining evolutionarily significant lineages that will ensure long – term population stability.

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THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT
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by

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OCTOBER – 2004

SUMMARY

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Population genetic theory predicts that small and isolated populations tend to loose genetic variations through genetic drift. (Lacy, 1987., Bouzat et al. 1998) As the human habitats continue to grow and rapidly encroach the wildlife habitats a better understanding of the population genetic structure of the wildlife is desirable for the development of management interventions that may circumvent their extinction possibilities. There are at least three biological reasons that make the preservation of genetic variation of wildlife populations one of the major goals of conservation biology. First the loss of genetic variation may increase the probability of population extinction through a decline in fecundity and viability, i.e. inbreeding depression. (Lacy 1987; Ralls, 1988; Meffe and Carroll, 1994; Frankham; 1995). Second populations with low levels of genetic variation, upon which natural selections can operate, may have reduced opportunities for future adaptation through evolutionary change(Lande and Barrowclough,1987; Fahrig and Merriam, 1994; Meffe and Carroll,1994). Third, the preservation of genetic variation may play a key role in identifying evolutionary significant units for conservation, i.e. genetically distinct populations of management concern (Meffe and Carroll, 1994).

During the past few years, there has been considerable debate concerning the possible effects of fragmentation, isolation and small population size on the genetic variability of bird populations. The high vagility of many bird species typically results in high levels of gene flow and thus little local genetic differentiation. (Barrowclough, 1980). Only a limited number of studies have demonstrated genetic effects as a result of fragmentation of continental bird populations. (Stangel et al. 1992; Haig et al 1993).

In this study firstly, the population genetic structure of the six munia (family Estrildidae) species of the subcontinent was evaluated and the levels of within species genetic variability assessed to determine the levels of genetic diversity within each species. Secondly the interspecific relationship between the six species was determined to estimate the levels of differentiation and relatioships between the species to reconstruct the phylogeny of the family Estrildidae in the subcontinent. The studies of within and between populations provided insights on speciation and a better understanding of the evolutionary trends It proved to be important for assessing prospective evolutionary potentials as well as for risk assessments and conservation strategies.

Advances in molecular techniques have led to an explosive increase in the studies of avian phylogeny. Polymerase chain reaction (PCR) amplifications of small amounts of DNA (nanogram), enable us to analyse genetic profiles without harming endangered birds. RAPD was chosen because it is expeditious and inexpensive and does not require a previous knowledge of the genome of species under study (Lynch and Milligan, 1994). Earlier comparisons showed that RAPD and isozyme results are very similar, with the RAPD technique revealing even higher amounts of variation (Baruffi et al., 1995).

Only six species of Estrildidae could be considered for the study . Two species belonged to the genus Estrilda or Amandava ; the Red munia *A.amandava* , and the Green munia *A.Formosa.* And the other four species belonged the genus Lonchura; the White backed munia, *Lonchura striata,* the Black headed munia *Lonchura malacca,* the Spotted munia *Lonchura punctulata,* and the White throated munia *Lonchura malabarica* Another species of Lonchura , *L.kelaarti* though chosen for the study was not obtained during the course of the work. With its preferred habitat of high range vegetation; the species has become highly localized and uncommon.

(Satish et al, 2003). *L.kelaarti* and *A.Formosa* are the two endemics of the family Estrildidae.

In the present study the two species of Estrilda , *A.amandava* and *A.Formosa* recognized by Kakizawa and Watada,1985 as belonging to the subgroup Estrildi of the group Estrildinae were analyzed for their within species genetic diversities and the between population relationships and variations. Owing to their coloured plumage the two estrilids have been grouped together. But a considerably high between species genetic distance have been observed compared to the passerines. Wolters, (1975-82) had placed these species in two different genera owing to the aberration in their characters. *A. amandava* is a widely distributed and well established species. The within population genetic diversity estimated does not show any levels of deterioration. Whereas, *A. Formosa* is an endemic with restricted distribution in central India. The pattern o f genetic diversity observed in the population of *A. Formosa* is consistent with the idea that recent fragmentation and isolation may have increased their local differentiation from other estrilids and decreased their within population genetic variability as a result of stochastic events associated with small population size and inbreeding. And inbreeding will consistently lead to a decrease in genetic variability over time. The study clearly indicates that the Green munia population is genetically unique and may be considered as an important evolutionary unit of conservation.

The four species of Lonchura belonged to the subgroup Lonchuri of the group Lonchurinae (Kakizawa and Watada, 1985; Mayr, 1968; Wolters, 1975-82; Delacour,1943). Commonly known as the mannikins they form a uniform group with somber plumage coloration which show no sexual color dimorphism. Considerably good within species genetic variation has been noted among the Lonchura species reflecting a continuous and abundance in distribution. The less degree of genetic differentiation among the Lonchura species is possibly related to the short period that these

populations have been isolated. It also indicates the relatively recent genetic differentiation of the group. Analysis of percentage polymorphism reveals *L.Malacca* as the most genetically diverse species. Hence assuming that the Malacca population is representative of "large" genetically diverse population s, then populations of the other three Lonchura species may be depauperate. *L.malabarica* or the Indian silverbill also exhibit a considerably good genetic diversity. The phylogeneic dendrogram reveals *L.punctulata* and *L. striata* as the direct descendants of the Indian silver bill, *L.malabarica.* The genetic distances of *L.punctulata* and *L striata* reflect their extremely low genetic differentiation and most recent speciation. A fifth species of Lonchura , *L.kelaarti* an endemic species of the Western Ghats could not be included in the present study owing to its sparse and restricted distribution. Yet the distribution of the species has not so far been reported as deteriorating. The species is distributed in the high rainfall moist deciduous and evergreen forests of the southwest India and the western ghats. This forest has been much reduced by commercial timber extraction and the construction of hydropower dams. Only moderate areas of seminatural forests remain except in the narrow strips of coastal plains where natural forests has been practically eliminated. With further reduction of their preferred habitat *L.kelaarti* could easily become vulnerable to extinction.

The results show that of the two groups of Estrildidae considered, the genus Lonchura with lower rate of genetic differentiation reflect more recent speciation and that the older species belong to the Amandava or genus Estrilda. This result adds extra weight to the hypothetical African origin of the Estrildidae as recognized by Kakizawa and Watada. (1985).The population structure of the endemic species *A.Formosa* and *L.kelaarti* reported in this study is consistent with the idea that fragmentation, isolation and small population size may be affecting their genetic variability. Therefore present status of the two endemics warrants further investigations.

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RECONSTRUCTION OF PHYLOGENY OF BIRDS OF THE GROUP ESTRILDIDAE BY USING DNA COMPARISONS AND DNA MARKERS

THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSPHY IN ZOOLOGY

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APPENDIX

APPENDIX

BUFFERS AND SOLUTIONS

1. **RBC Lysis Buffer:**

   - breaks up the cell and releases DNA and other contents

   D.D Water--------------------100ml

   KCl (74.55)-------10mM------ 0.07455g

   NH_4Cl (53.49)---150mM------0.80235g

   EDTA(disodium)---0.1mM---0.02ml (20µl of 0.5 M stock)

   Weighed out KCl and NH_4Cl, dissolved in about 80 ml Double Distilled water- then added 20µ l of 0.5M EDTA, made up to 100 ml and autoclaved.

2. **T.E Buffer** (Tris pH-7.6, EDTA- pH-8)

   Water-------------- 50ml

   Tris.Hcl-------------10mM ----0.12g

   EDTA (disodium)---1mM ---0.2ml (200µl of 0.5M stock)

1M Tris. HCl (pH 7.6)

dd Water---10ml

Tris Base (eq.wt.121.14g)-----------1.211g

Conc.HCl -----0.6 ml

Dissolved 1.211g of Tris Base in 7.5ml distilled water, added 0.6ml of conc.HCl and made up the volume to 10ml.

0.5 M disodium EDTA (pH 8)

dd Water--------------100ml

disodium EDTA----------------18.6g

Added 50 ml water to disodium EDTA .Then bring the pH to 8 by adding Na OH flakes. After dissolution made up the solution to 100ml.

Buffer:

1MTris. HCl (pH 7.6) -----0.01ml (10µl)

0.5M EDTA(disodium)------------0.2ml (200µl)

Made up to 50 ml with D.Dwater and autoclaved.

3. Proteinase K.

Water ----1ml

Proteinase K----20mg

Dissolved and kept chilled in -20°c.

4. **SDS 10%**

- aids in nuclear lysis and release of DNA indicated by a dramatic increase in viscosity.

Water—5ml

SDS---- 500mg (0.5g)

Dissolved the weighed out SDS in water without shaking much to prevent frothing.the soluion is no5t autoclaved and not refrigerated.

5. **Saturated Nacl soln.**

- prevents dissolution of DNA and aids in salting out.

Prepared 5M soln ie 30g/100ml or dissolved until saturation on a magnetic stirrer.

6. **Chloroform:Isoamyl (24:1)**

- aids in deproteinisation of DNA .

Mixed the two in proper proportions with great care in deodourizer chamber.

7. **Isopropyl Alcohol**

(0.6 times the volume of the supernatant)

Or Double distilled Ethyl Alcohol (2 times the volume of the supernatant.) may be used.

- to precipitate the DNA.

8. **70% Ethyl Alcohol** (prepared fresh)

- for washing to remove any remaining salts.

9. **Phenol**

Tris saturated (pH 7.5)

10. **Sodium acetate**

M (pH 5.2)

11. **DNA tracking dye (6x)**

Bromophenol blue 0.25%

Sucrose 40% w/v.

NB 4958

12. **Ethidium Bromide.**

10mg /ml

13. **10 X TBE Buffer (pH 8.5)**

Tris base -------- 54g

Boric Acid -------------27.5g

Disodium EDTA ------- 4.75g

d.d.Water -------------500ml

- Dissolved the above salts in 500ml d.d.water and autoclaved.