ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF BIOLOGY

ANALYSIS OF THE GENETIC DIVERSITY OF POPULATIONS OF *Rana angolensis* BOCAGE 1866 USING THE ISSR MARKER

A Thesis Presented to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Genetics)

Michael Hailu

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Declaration:

I, the undersigned, here by declare that this thesis is my original work and that all sources of materials used for the thesis have been duly acknowledged.

Name:_ Michael Hailu_____

Signature: _____

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Analysis of genetic diversity of population of *Rana angolensis* (Bocage, 1866) in Addis Ababa using the ISSR marker

By

Michael Hailu

Approved by the Examining Board:

Name	Signature
_____	_____
Examiner	
_____	_____
Examiner	
Endashaw Bekele (Professor)	_____
Research Advisor	
_____	_____
Chairman	

June, 2008

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May God bless you all!!

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List of Abbreviations

AFLP-Amplified Fragment Length Polymorphism

AMOVA-Analysis of Molecular Variance

GAA- Global Amphibian assessment

GD- gene diversity

H'- Shannon-weaver diversity index

ISSR-Inter Simple Sequence Repeat

NPL-number of polymorphic loci

NJ-Neighbor Joining analysis

PCO-Principal coordinated Analysis

PP (%) -percent polymorphism

RFLP-Restriction Fragment Length Polymorphism

RAPD-Random Amplified Polymorphic DNA

SNPs- Single Nucleotide Polymorphisms

UPGMA – Unweighted pair group method with arithmetic mean

Abstract

Four populations of the common river frog, *Rana angolensis* Bocage 1866*,* in the surroundings of Addis Ababa (Akaki, Entoto, Gulele and Kotebe), Ethiopia, were studied with the sample size of 10 for each. Inter Simple Sequence Repeats (ISSRs) as a molecular marker was used to assess genetic diversity, both within and between populations, using three di-nucleotide ISSR primers. A total of 29 clear and reproducible bands were amplified from the three ISSR primers. The over all gene diversity and percent polymorphisms were found to be higher in Akaki than in other populations (0.22). The Shannon-weaver diversity index also confirmed the existence of higher diversity in Akaki *Rana* populations than the other populations used in the present study. Furthermore, partitioning of the Shannon-weaver diversity showed that the majority of the variations were observed within populations of the Akaki and Entoto (68.89% and 55.17% respectively). Similarly, AMOVA demonstrate highly significant (P=0.00) genetic differences within each populations than among populations of *Rana angolensis*. Of the total variation, 27.25% was attributable to among populations, while 72.75% to within population variation. Both UPGMA and neighbour joining trees were constructed for each individual and population using Jaccard's similarity coefficient. The trees do not clearly indicate the four distinct groups which are based on populations of area origin. The PCO analysis has also recovered the UPGMA and neighbour joining trees and each population do not appear to form a clear cluster.

. **Key words:** genetic diversity, *Rana angolensis* populations, ISSR, Addis Ababa

(Berger *et al,* 1998; Daszak *et a*l, 2004), but in many cases the causes of decline are unknown (Stuart *et al*, 2004). Amphibians are capable of leading a double mode of life. Some amphibians, like those in the Genus *Arthroleptis*, spend their entire lives out of aquatic environment. However, most amphibians spend most of their lives in water. They are highly developed chordates characterized by a firm dorsal rod, notochord, which forms the basis of spinal column in the evolutionary history of the group and in the development of the individual organism. The presence of a notochord in the early stage of development, which is later replaced by a vertebral column, makes amphibians to be grouped in vertebrate together with fishes, reptiles, birds, and mammals (Mertens, 1960). Skeleton of amphibians are mostly bony with varying number of vertebrae (Mertens, 1960).

Currently in Ethiopia, eight families consisting of 24 genera and 63 species of amphibians have been recorded. Nearly 40% are endemic, with four species placed in the endangered category of the IUCN red list. Many more are listed in near threatened and vulnerable categories (IUCN, 2007). The approximately 250 extant species of true frogs (*Rana*) are found throughout much of the world, with the major exceptions being the Polar Regions, most of Australia, and the temperate regions of South America. About one-quarter of the species of *Rana* are found in the Americas, with the largest concentration in the southern United States and Mexico. In the New World, species of *Rana* are found from Alaska and Canada south throughout the continental United States and all of Middle America to northwestern Peru on the west side of the Andes and to eastern Brazil and northern Bolivia on the east side of the Andes (Cochran, 1970). Collectively, these species are found in almost all of the major biotic provinces that are inhabited by frogs—tundra, temperate coniferous and deciduous forests, grasslands, deserts, brackish-water marshes, freshwater streams and lakes, semitropical cloud forests, and tropical rain forests (Cantino, 1999).

The common river frog, *Rana angolensis* Bocage 1866, has a wide distribution in Africa especially in the sub Saharan Africa. It is active through out the year. In Ethiopia, it has been found in a wide range of climatic and ecological ecosystems. It requires permanent water surrounded by dense herbaceous vegetation (Largen, 2001). The species finds such conditions in grassland on central plateau in the forests of south western Ethiopia, where it has been recorded at altitudes of about 1000-3000 m (Largen, 2001). It is far from certain that these populations are

contiguous with further south in Kenya and Uganda, though there has never been any suggestion that they might be taxonomically distinct. To date, no molecular or phenotypic diversity data have been generated for the species in this country. Despite the status of population structure indicates that it has been successful in colonization of habitats and surviving natural selection, it has been generally accepted that global amphibian population are declining as a result of many causes. Therefore, studies with regard to the diversity and population structure of this species will be vital for future scientific studies as well as for devising successful conservation strategies.

We can use the study of genetic variation to examine variations between members of the same species. We can compare the genetic composition of members of different species even over a wider taxonomic range (Dale and Schantz, 2002). Studies on genetic variation are one of the most important parameters for conservation of genetic resources. Different molecular markers can be used for direct measurements of genetic variation. Ultimately, comparison of complete genome sequences is the only way of detecting all the possible types of variation that may occur. However, we need quicker methods which are targeted at certain types of changes (Dale and Schantz, 2002).

Inter Simple Sequence Repeats (ISSR) has been successfully employed to assess genetic diversity within and between populations in several plant and animal species. As a dominant marker, ISSR targets simple sequence repeats (microsatellites) that are abundant through out the eukaryotic genome and that evolve rapidly. As a consequence, ISSR amplification reveals a much larger number of polymorphic fragments per primer than does other marker systems like Random Amplified Polymorphic DNA (RAPD). The technique also does not require prior knowledge of DNA sequence for primer design, and has advantages similar to those of RAPDs (Kantety *et al*., 1995). Furthermore, ISSR is preferable in terms of the polymorphic bands detected per primer and the reproducibility involved. The general objective of this study was, therefore, to analyze the genetic polymorphism and population genetic structure of common river frogs (*Rana angolensis*) in river basins in the surroundings of Addis Ababa.

2. Literature Review

2.1 Taxonomy

Amphibians are a diverse class of vertebrates that are grouped into three orders of approximately 58 families, 389 genera, and more than 6000 species (Frost, 2007). The number of species and genera are growing from time to time as new species are discovered. Existing taxonomy of amphibians are based mainly on morphological characters such as internal anatomy of the skeleton, larval characters, developmental patterns, nature of mating (amplexus) and pupil shape. Ecological researches also provide clues to separate sibling species that were previously overlooked. But by the end of the twentieth century, cytological and molecular techniques provided support for some arrangements based on morphological features (Beck and Slack, 2001).

The genus *Rana* belongs to the subfamily *Raninae* in the family *Ranidae*. There are approximately 754 species and sub-species in this genus in the tropical and sub-tropical regions of Africa, Asia, Australia and South America, with the largest number in Africa, which is usually considered the centre of origin of the genus. Most of the species are diploid (2n=24) and rarely, tetraploids (2n=48) are found.

Because several species of *Rana* are common throughout much of the world, several species of *Rana* have served as research subjects for a broad array of studies in evolution, ecology, development, genetics and physiology. Given the large amount of comparative biological information available among species of *Rana,* this group has great potential for placing a wide range of biological studies in an evolutionary framework, as long as phylogenetic estimates for the group are available. At least six species of the Old World *Rana* are thought to have become extinct in historic times, and several of the remaining species have undergone serious declines and are threatened with extinction. In addition, several Old World tropical species have not yet been described.

Rana has been tentatively subdivided into several sub-genera and species groups with some members being elevated to separate genera (Dubois, 1986). One sub-genus, *Rana,* is distributed throughout Eurasia, America, and parts of Africa and South America, and contains at least 10

species (Frost, 2007).

Within eastern Africa, this sub-genus appears to contain at least four species: *Rana angolensis* Bocage 1866, *Rana nutty* Boulenger 1896*, Rana fuscigula* Bocage 1866 *and Rana oxyrhynchus* Günther 1893. Characters which distinguished these species (but have never been seriously analyzed) may be summarized a follows: *Rana angolensis* appear to exhibit at least one obvious picture, which may be of diagnostics value: adult males have heavily pigmented longitudinal pleats in the gular skin, bordering the margins of the lower jaw (Largen, 2001). *Rana nutty*, with a reddish brown colour has a slim body shape and dark mark across tympanum. *Rana fuscigula* is often distinguished by a greenish colour, robust body shape and wide and straight dorsolateral folds. *Rana oxyrhynchus* has a large inner metatarsal tubercle, a single nuptial pad, external vocal sacs and toe tips pointed (Largen, 2001).

2.2 Morphology and reproduction

Rana angolensis, a slim, long-legged, green or brownish frog usually with well-defined, pale-bordered, oval or round dark dorsal spots; brown or black stripe on upper jaw; white or cream below; well-defined, pale dorso lateral ridges that are not inset at the posterior end; dark dorsal spots may be reduced or absent in young; during the breeding season, adult males have swollen, darked thumb bases and usually have vestigial oviducts; adults generally are 5-9 cm in snout-vent length, sometimes up to 11 cm (Figure 1.1) (Stebbins, 1985). The dorsum sometimes has few or no dark dorsal spots or much dark pigment between the dark spots (Collins, 1991).

Breeding often peaks when water temperature reaches about 10^{o}C. At a particular site, egg deposition generally occurs within a span of about 10 days. Egg masses include several hundred to thousand ova. Aquatic larvae usually metamorphose in summer. larvae may overwinter in some areas. Females are sexually mature usually in two years in most areas, three years in high elevation populations. Density of egg masses often reaches a few hundred per ha(sometimes >1000/ha) in favourable habitat,.

Fig 1.1 *Rana angolensis*

2.3 Distribution

The common river frog (*Rana angolensis*), as currently conceived, has a huge geographical range in the savannas of Africa from Nigeria to Somalia and hence Southwards to Angola, Namibia, and the Cape Province of South Africa (IUCN, 2007). It dwells in permanent aquatic habitat surrounded by dense herbaceous vegetation. The species finds such conditions in grassland on central plateu and in the forests of southern Ethiopia. It lives in altitudes ranging from 1000-3100m. *Rana angolensis* specimens have been collected at elevations of about 400-2500 m in western and central Ethiopia, where it is widespread and common in habitats that include both moist and savannah, montane grassland and forest margins. Such an ecological profile is typical of most African aquatic environments, and the species, therefore, being found to occur in many range states (Figure 1.2). The status of population structure indicates that it has been successful in colonization of habitats and surviving natural selection. However, it is generally conceived that populations of this species have undergone a dramatic decline during the past 20 years (GAA, 2007). The causes of the decline have not been studied exhaustively, and the status of the remaining regional populations is poorly known due to the scarcity of research information.

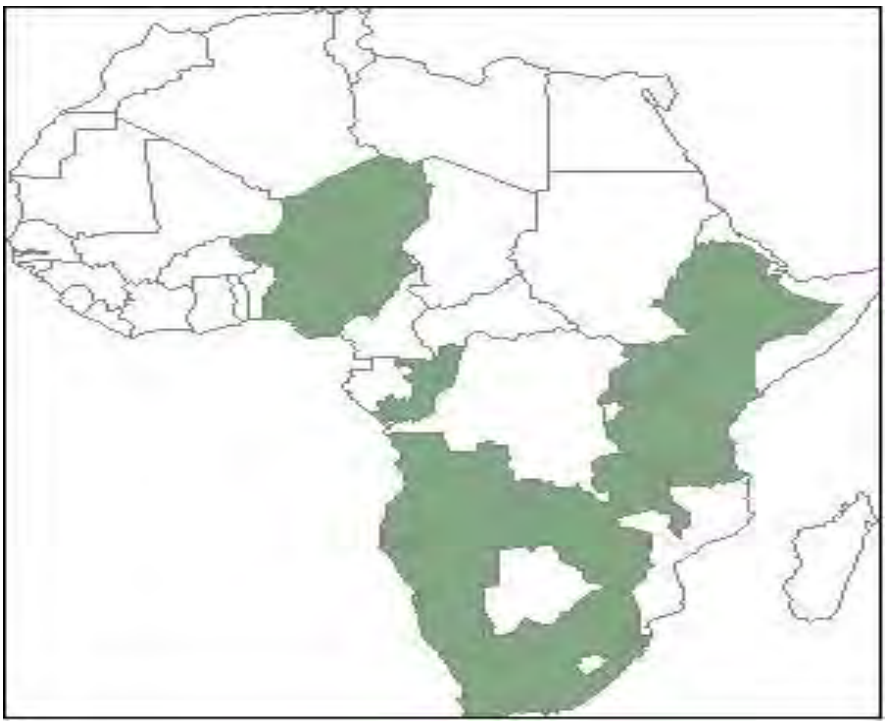

Fig 1.2 Map showing the current distribution of *Rana angolensis* in the sub Saharan Africa (shaded area) (Source: Global Amphibian Assessment, GAA, 2007)

2.4 The Global Amphibian decline

At the First World Congress of Herpetology in 1989, researchers began gathering largely anecdotal evidences that a global pattern of population decline and species losses were occurring among the world's amphibians (Stuart, 2004). Herpetologists from several continents reported drastically dwindling and even completely extirpated amphibian populations that had been observed thriving only a few years earlier. Since then, increased evidence and analysis of population trends have suggested that massive amphibian declines are occurring at local scales in many regions around the world, including North and South America, Africa, Asia and Australia. Moreover, analysis of hundreds of amphibian populations worldwide has indicated a global amphibian population decline that may have been occurring for several decades. There is now a consensus among scientists that alarming rates of amphibian declines have occurred.

Amphibians are integral components of many ecosystems, and in some habitats their combined biomass exceeds that of all other vertebrates combined (Frost, 2007). Due to their important role

in the food web of many communities, declines in amphibian populations could have an important impact on other organisms. For instance, overharvesting of wild Indian bullfrogs (*Rana tigrina*) in India and Bangladesh for the frog leg trade led to an overabundance of insect pests that are the frogs' typical prey (Arachchi, 2004). Adult amphibians are thus important carnivores in many ecosystems, as well as prey species in others. Larval amphibians (tadpoles) can be important herbivores as well as prey.

The most important question is whether these declines are the result of natural population fluctuations or the result of human-induced disturbances to the environment. Declines of amphibian populations due to habitat loss and introduced exotic predators have been documented and are obviously the result of human causes (Camargo and Heyer, 2005). However, more alarmingly, researchers have also documented drastic declines in relatively pristine areas such as nature reserves (Camargo and Heyer, 2005). Though a common cause among these unusual declines has yet to be identified, there is evidence that they are also the result of human perturbations (Table 1.1). Because of their complex life histories and ecological interactions unique among vertebrates, amphibians may be good bioindicators of subtler forms of environmental degradation. Their life histories involve eggs and larvae in aquatic systems and adults in terrestrial systems, making them vulnerable to degradation of both types of habitats. The adults of many amphibian species spend their lives against or in a substrate such as mud, sand, leaf litter, and water, making them vulnerable to toxic contamination of that substrate (Williams, 2001). With skin that is permeable to water, electrolytes and gaseous molecules, individuals at all life-history stages are vulnerable to environmental contamination. Moreover, amphibian eggs are covered only by a layer of gelatinous material, resulting in relatively direct exposure to the environment and making recruitment of new individuals into a population very susceptible to environmental conditions (Williams, 2001) (Figure 1.3). Vulnerability of amphibians to subtler forms of environmental degradation has led researchers to suggest that amphibian populations are declining due to local causes such as toxic contaminants and acid precipitation, as well as due to global phenomenon such as climate change and increased ultraviolet radiation from ozone depletion. Amphibians weakened by the above factors may have lower rates of successful reproduction or be more susceptible to pathogens, which have also ravaged amphibian populations (Stuart *et al*., 2004).

Relatively, little knowledge available about the population dynamics of amphibian populations worldwide continue to challenge researchers. Understanding the extent of the problem and its nature requires an understanding of how local populations naturally fluctuate, and how local factors affect the dynamics of these populations

Table 1.1. Habitat preferences and biogeographic affinities of rapidly declining amphibian species. (Source, Global Amphibian Assessment, GAA, 2007)

↓ denotes significantly lower than average, † denotes Significantly higher than average

Habitat preferences	Total number of species (%)	Number of rapidly declining species (%)	Number of enigmatic-decline species (%)
Forest	4699 (81.8)	365 (82.6)	187 (90.3)†
Shrubland	814 (14.2)	47 (10.6)*↓	14 (6.8)↓
Grassland	953 (16.6)	81 (18.3)	39 (18.8)
Flowing water	2650 (46.1)	277 (62.7)†	164 (79.2)†
Marshes/swamps	760 (13.2)	43 (9.7) ↓	14 (6.8)↓
Still water bodies	2030 (35.3)	107 (24.2)↓	28 (13.5)↓
Artificial habitats	1304 (22.7)	40 (9.0)↓	22 (10.6)↓
Tropical habitats	3392 (59.1)	212 (48.0)↓	79 (38.2)↓
Tropical habitats	2714 (47.3)	251 (56.8)†	155 (74.9)†
Biogeographic realms			

Afrotropical	951 (16.6)	28 (6.3)↓	1 (0.5)↓
Australasian/Oceanic	561 (9.8)	36 (8.1)	23 (11.1)
Australia and New Zealand	219 (3.8)	32 (7.2)†	23 (11.1)†
Indomalayan	938 (16.3)	59 (13.3)	1 (0.5)↓
Nearctic	331 (5.8)	24 (5.4)	9 (4.3)
Neotropical	2,825 (49.2)	279 (63.1)†	174 (84.1)†
Palearctic	451 (7.9)	34 (7.7)	

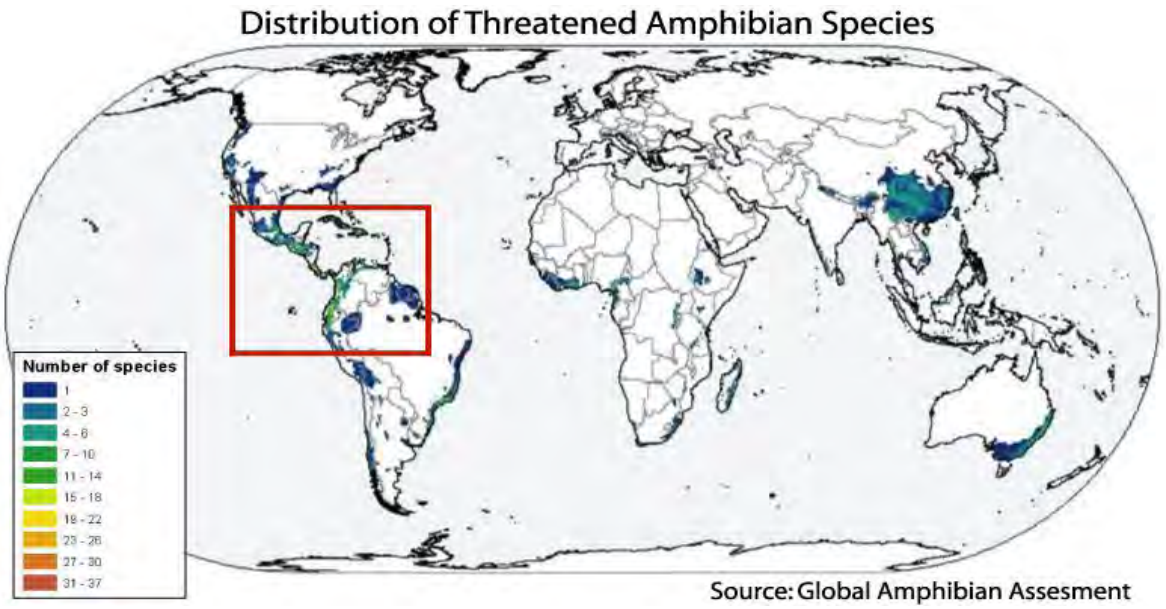

Fig. 1.3. Distribution of globally threatened amphibian species by Region. (Source, Global Amphibian Assessment, GAA, 2007)

2.5. Biodiversity and identification of hotspots

Understanding patterns of biodiversity may be the key to conserving remaining species, especially in tropical areas of the world. Biological diversity tends to be concentrated in "hot spots" corresponding to areas with historically high rates of geological change, rather than uniformly distributed across a given habitat or zone (Williams 2001). For example, tropical diversity is concentrated in South America, in the Indo-Malaysian region, and in the Eastern Arc Mountains of Africa, areas whose geological histories are extremely complicated. Biological diversity has been shaped by millions of years of interactions between speciation and adaptation. Using the information available from phylogenetics and vicariance biogeography, we can identify those areas of endemism and high diversity that will ensure the preservation of evolutionary potential, make predictions about the characteristics of rare or poorly known species and begin to answer questions about the susceptibility of species and ecosystems to environmental perturbations. Politicians and scientists now agree that a priority list of global centers for preservation of biological diversity is required. Diversity has generally been measured only in terms of species richness, or in the form of indices combining richness with abundance or, more recently, based on indices that contain cladistic classifications of taxonomic distinctness. This measure of taxonomic diversity, when coupled with detailed knowledge of distribution, can be used in modified analyses of the type previously developed as 'critical fauna's analyses' or 'network analysis' (Faith, 1992).

For practical planning, two basic rounds of analyses are required. Firstly, recognition of global priority areas by taxic diversity techniques. Secondly, within any such area, analysis to identify a network of reserves that contains all local taxa and ecosystems (IUCN, 2007). The assessments of amphibian diversity require exploration of previously unvisited areas, comprehensive surveys of poorly known areas, and revisiting of localities that have not been assessed in the last decade. These biodiversity surveys undoubtedly will result in the discovery of many new species, but will also provide ecological, behavioural and genetic data to assess species complexes and levels of non-morphological differentiation. Tropical and poorly known areas should be a high priority of the biodiversity surveys. Furthermore, the Global Amphibian Assessment (GAA) identified a large number of species as Data Deficient, meaning that the conservation status of their populations cannot be determined from the current available data. It is also important to assess

the current population status and potential threats to Data Deficient species. It is likely that some of those species are already threatened with extinction.

2.6. The phylogenetic framework and the recognition of unique lineages

Over the last century, the assessment of amphibian evolutionary relationships has not kept pace with the description of amphibian diversity. Hence an evolutionary and phylogenetic framework is needed to evaluate amphibian declines. A phylogenetic framework of amphibian relationships, primarily derived from molecular data, recently presented (Frost *et al.* 2007) should expedite this process. This framework is critical, if we are to assess the causes of the declines or predict which species and lineages are at a higher risk of decline and extinction. Because of the large number of undescribed species and the existence of morphological conservative species complexes, it is difficult to determine the amount of genetic diversity that is being lost through reported declines and extinctions. If the current amphibian declines have a historical component, i.e., if they are phylogenetically constrained, then conservation efforts must centre on the more susceptible clades. However, if a comprehensive phylogenetic analysis shows that the amphibian declines are randomly distributed among amphibian clades and lineages. Therefore, efforts need to focus on the causes of decline throughout the entire class (Frost, 2007).

As species represent history, special attention should be given to the conservation of phylogenetic diversity. One way to approach this issue is to be attentive to maximizing total diversity across nodes on a phylogenetic tree (Faith 1992; Wall *et al*., 1999), both globally and, to some degree, locally. In prioritizing taxa and habitats for conservation efforts, we seek to maximize both representation and persistence of diversity. The production of robust phylogenetic hypotheses for all species of amphibians is also the focus of the current AmphibiaTree project. The efforts mentioned above will permit the recognition of narrowly endemic clades with very long branches (i.e., they are long-branch taxa with no close relatives). Identification of these clades and lineages merits special consideration in conservation efforts (Heyer, 2005).

2.7 Conservation genetics

Genetic diversity is recognized as a fundamental component of biodiversity and its protection should be incorporated into conventions and policies to protect amphibians. Direct assessment of genetic diversity is often given low priority and the assumption made that protection of diversity at or above the species level will de facto protect the underlying genetic and evolutionary diversity. However, this assumption is rendered suspect by the increasingly frequent detection of cryptic species (Moritz, 1998; Camargo *et al*., 2005; Heyer *et al*., 2005). Conservation genetics is the application of genetics to conserve species as dynamic entities capable of coping with environmental changes. It encompasses genetic management of small populations, resolution of taxonomic uncertainties, defining management units within species, the use of molecular genetic analyses in forensics and understanding individual species biology. It deals with the genetic factors that affect extinction risk, and genetic management regimes required to minimize these risks.

Many amphibian populations have very small effective population sizes, commonly less than 100 (Funk *et al*., 1999). This makes amphibian populations especially susceptible to loss of genetic diversity by random drift, i.e., the random loss of alleles, and ultimately to the effects of inbreeding depression and high genetic load (Frankham *et al*., 2002). Conservation measures for declining amphibian populations will need to take account of this population structure, especially in already fragmented landscapes, where the risks of population isolation are increasing with continuing fragmentation. Both phylogeographic and phylogenetic patterns have roles in conservation biology at different levels, from evaluations of heterozygosity in threatened populations to analyses of population structure and intraspecific phylogeography to species-level issues and higher-level phylogeny. Analyses of genetic diversity in rare species occasionally identify one or more populations that are genetically distinct from other populations by virtue of either unique alleles or organellar genomes or allele frequencies. In some cases, these populations or clusters of populations may warrant special management consideration because they represent unique genetic and, potentially, ecological components of species (Soltis and Gitzendanner, 1997).

2.8. Systematics as baseline for nature Conservation

The phylogenetic framework of amphibian diversity will serve to analyze the effects of multiple factors affecting the planet earth. Biodiversity surveys can identify how climate change is affecting and transforming the microhabitats by the changes in amphibian composition at any given site and by the decline of amphibian populations (Camargo and Heyer, 2005). Furthermore, the predictive power of phylogenetic hypotheses will suggest how other species (e.g., closely related species), may be affected by similar environmental changes. The same can be said about environmental contaminants and the effect that pollutant may have in amphibians, both in their aquatic as well as their terrestrial phase of life. Solid evolutionary hypotheses for amphibians are also critical to understand both susceptibility and resistance to diseases, parasites, and pollutants (Camargo and Heyer, 2005). Given the current declines and the potential role of emerging infectious diseases in the declines, it is critical to understand the spread and reach of the diseases and other threats within a phylogenetic framework. Understanding the evolutionary history of amphibians is critical during the decision making process to select and determine which among the areas and habitats are to be protected. Clearly, areas of high endemism, high diversity, and unique lineages will be identified in the resulting evolutionary hypothesis and will be critical in site planning. The distributional patterns resulting or enhanced by the biodiversity surveys will be the baseline data for any attempt to make reintroductions.

2.9. Marker systems and their application in genetic diversity analysis

2.9.1 Morphological markers

A Morphological marker is a marker system based on phenotypic appearance. It is the earliest genetic markers used for assessment of variation and still has great importance. Moreover, morphological characters are simple to score and inexpensive too. They represent several section of the genome, if several characters are used.

Amphibians lack such definite ectodermal structures as hair, feathers, and scales; therefore taxonomist must rely on other characters. Other disadvantages of this approach are the influence of the environment, extra time and resources needed for evaluation. Moreover, some morphological traits are observed to be very plastic in nature, which could be easily affected by

environmental changes and affect the exact relationships of populations (Harewood, 1995). The choice of characters thus needs to consider these problems.

2.9.2 Biochemical markers

Biochemical markers are markers based on protein enzyme polymorphisms through electrophoretic separation of protein molecules. A tissue extract is prepared and electrophoresed on a non-denaturing starch or polyacrylamide gel. The proteins of this extract are separated by their net charge and size. After electrophoresis, the position of a particular enzyme in the gel is detected using standard procedures. Depending on the number of loci, their state of homo-or heterozygosity, and the enzyme configuration (i.e., the number of separable units), from one to several bands are visualized. The positions of these bands can be polymorphic and can be considered as informative loci (Weising *et al*., 2005).

The main advantages of protein enzyme markers are their codominant inheritance and the technical simplicity and low cost of the assay. Disadvantages include the restricted number of suitable allozyme/isozyme loci in the genome, the requirement of fresh tissue and limited variations (Weising *et al*., 2005).

2.9.3 Molecular markers

Molecular markers are based on naturally occurring polymorphisms in DNA sequences (i.e.: base pair deletions, substitutions, additions or patterns) (Gupta, 1999). Molecular markers are better than morphological and biochemical markers, because they are abundant throughout the genome, completely independent of environmental conditions and can be detected virtually at any stage of the organism's development. Molecular markers can be applied in genetic mapping, marker assisted selection (MAS), population genetics and molecular systematics (Weising *et al*., 2005).

In recent years, different marker systems such as Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNAs (RAPDs), Sequence Tagged Sites (STS), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) or micro-satellites, Single Nucleotide Polymorphisms (SNPs) and others have been developed and

applied to a range of plants and animals (Weising, 2005). The relative advantages and disadvantages of these techniques are highly summarized below.

2.9.4 Restriction Fragment Length Polymorphism (RFLP)

In Restriction Fragment Length Polymorphism, a molecular marker based on the differential hybridization of cloned DNA to DNA fragments in a sample of restriction enzyme digested DNAs, the marker is specific to a single clone/restriction enzyme combination. In RFLP analysis, DNA is extracted from organism of interest, and digested with one or more restriction enzymes. The resulting fragments are separated according to size by gel electrophoresis. The gel is southern-blotted on to a membrane, and one or more specific fragments are visualized by blot hybridization with a labeled probe (Weising *et al*., 2005) .

The main advantages of RFLP markers are their co-dominance and high reproducibility. However, the drawbacks as compared with PCR- based techniques is that the requirement of relatively pure and intact DNA (Weising *et al*., 2005), technically laborious process and comparatively high expenses.

2.9.5 Random Amplified Polymorphic DNA (RAPDs)

Randomly Amplified Polymorphic DNA; a molecular marker based on the differential PCR amplification of a sample of DNAs from short oligonucleotide sequences. Random amplified polymorphic DNA (RAPD) markers were first described in 1990 (FAO, 2003). RAPD was the first PCR based molecular marker technique developed and it is by far the simplest. Short PCR primers (approximately 10 bases) are randomly and arbitrarily selected to amplify random DNA segments throughout the genome. The resulting amplification product is generated at the region flanking a part of the 10 bp priming sites in the appropriate orientation. RAPD often shows a dominant relationship due to primer being unable to bind (show 3:1 ratio, unable to distinguish between homozyogotes and heterozygotes) (Yin *et al.,* 2001).

Advantage of RAPD is its technical simplicity, paired with the independence of any prior DNA sequence information. However, its dominant nature and sometimes the problems of reproducibility are its main disadvantage (Weising *et al*., 2005).

2.9.6 Amplified Fragment Length Polymorphism (AFLPs)

In the mid 1990's, another PCR-based method of molecular markers was described, giving rise to amplified fragment length polymorphism (AFLP) markers. With this technique, the DNA treated with restriction enzymes is amplified with PCR. It allows selective amplification of restriction fragments giving rise to large number of useful markers which can be located on the genome relatively quickly and reliably (FAO, 2003).

AFLP can be used to distinguish closely related individuals and can also map genes. Like that for RFLP, high quality DNA is also required. AFLP is an extremely sensitive technique and the added use of fluorescent primers for automated fragment analysis systems, and sophisticated software packages to analyze the biallelic data, makes the AFLP well suited for high throughput analysis (Farooq and Azam, 2002).

The major advantage of the AFLP technique is generation of a large number of polymorphism (Geleta, 2007). The fact that no sequence information is required and that the PCR technique is fast with a high multiplex ratio makes the AFLP very attractive choice (Rafalski *et al.,* 1996).

2.9.7 Single Nucleotide Polymorphisms (SNPs)

SNPs are single base pair positions in the genomes of two or more, at which different sequence alternatives (alleles) exist in populations (Weising *et al.,* 2005). In recent years, single nucleotide polymorphisms (SNPs), i.e. single base changes in DNA sequence, have become an increasingly important class of molecular marker. The potential number of SNP markers is very high, i.e., that it should be possible to find them in all parts of the genome, and micro-array procedures have been developed for automatically scoring hundreds of SNP loci simultaneously at a low cost per sample (FAO, 2003).

2.9.8 Inter Simple Sequence Repeats (ISSR) markers in genetic diversity analysis

The inter simple sequence repeats (ISSR) are a new kind of molecular marker and glorified RAPD involving PCR amplification of DNA by a single primer, (16-18) bp long, composed of

repeated sequence anchored or nonanchored at the 3' or 5' end by 2-4 arbitrary nucleotides (Zietkiewicz *et al*., 1994). They are easy to handle, highly informative and repeatable.

ISSRs are now being applied for assessment of genetic diversity in various plant and animal species (Tesfaye *et al*, 2006; Girma, 2007; Pomper *et al.,* 2003) and in determining genetic diversity and phylogenetic relationships (Joshi *et al.,*2000).

3. Objectives

3.1 General objective

The general objective of the present study is to provide information that could be implemented in the analysis of some evolutionary factors operating in *Rana angolensis* and future conservation or utilization strategies.

3.2 Specific objectives

- To perform ISSR analysis in order to estimate the magnitude and pattern of genetic diversity in *Rana angolensis*.
- To identify the level of genetic diversity within and among population of the species
- To provide baseline data for further analysis and utility of this frog species.

4. Materials and Methods

4.1 The study area

Representative samples of populations used in the present study were collected from four sites in and around Addis Ababa. Three of the collection sites were localized in river systems, while the fourth has been located in an isolated water body (pond). Two of the study sites were located at the upstream and down stream of the Kebena River. One site was at Entoto and the other at Akaki. Specimens were also collected at Kotebe from a small tributary river that drains into the Welgemo River. The smaller tributary was chosen due to its richness in certain amphibian fauna as it has been affected little by human interference. The fourth site was at Gulele, an isolated permanent pond, which was prone to severe anthropological disturbances. This site had a relatively less number of *Rana angolensis* populations while there was more number of *Xenopus clivii*.

4.2 Specimen collection

Ten individual frog samples were collected from each of the four sites mentioned above. Sampling was done randomly with approximately 6 m distance from each other. Capturing was done using a net attached to a long wood bar to allow sampling as deep as 4m. Captured frogs were immediately transferred into a vessel filled with warm water (40 to 50 centigrade's), which will anesthetize and eventually kill the frogs. A low amount of muscle tissue was then taken from the leg of each frog sample and transferred into a sterile eppendorf tube containing 96% ethanol. In addition to muscle, liver tissues were also taken and preserved in separate tubes containing 96% ethanol. The remainder of the frog samples was then preserved in large jars containing 70% ethanol.

4.3 DNA extraction

DNA was extracted using the Animal DNA extraction kit (Avegene), which uses Proteinase K and a Chaotropic salt, guanidine hydrochloride to lyse cells and degrade protein and eluting the purified DNA with an elution buffer. Expected yield of genomic DNA was up to 50 micrograms and purified DNA with approximately 20 to 30 kilo base pairs was assumed suitable for PCR.

4.4. Test gel and electrophoresis

An agarose gel (100ml, 1xTBE and 0.98g agarose) was prepared and 2 µl each genomic DNA samples with 6µl loading dye was loaded on to the gel and electrophoresed at constant voltage of 80V for 45 minutes. The gel was stained for 30 min with 50 µl ethidium bromide (10mg/ml) after well mixed with 450 ml distilled water and then de-stained for 30 minutes with distilled water. Gel picture was taken under UV transilluminator by BiodocAnalyse 2.0 with digital canon camera.

4.5. Primer selection and optimization

A total of six ISSR primers obtained from the University of British Colombia (primer kit UBC 900) were used for the initial testing of variability and reproducibility. One individual was selected from each population to screen the primers with 1:5 dilutions and finally three primers were selected and these include 2 di-nucleotides and 4 tetra- nucleotides repeat motives (Table 4.1) based on polymorphism and reproducibility.

Table 4.1:- List of primers, annealing temperature and sequence. All the primers are high performance liquid chromatography purified and designated as 'H'

Primers	**Annealing temperature**	**Sequence**
811- H	48^{o}C	5'-GAG AGA GAG AGA GAG AT-3'
818- H	48^{o}C	5'-CAC ACA CAC ACA CAC AG-3'
827- H	48^{o}C	5'-TCT CTC TCT CTC TCT CG -3'
826-H	45^{o}C	5'-TGT GTG TGT GTG TGT CG -3'
809-H	48^{o}C	5'-AGA GAG AGA GAG AGA AT-3'
816-H	48^{o}C	5'-GAC CAG GAC CAG GAC AG-3'

4.6. PCR and gel electrophoresis

The polymerase chain reaction was conducted in Biometra 2000 T3 Thermo cycler. PCR amplification was carried out in a 25 µl reaction mixture containing 1µl template DNA, 13.2µl H_20, 5.6µl dNTP (1.25mM), 2.6µl Taq buffer (10xThermopol reaction buffer), 2.0µl $Mgcl_2$ (2mM), 0.4µl primer (20pmol/µl) and 0.2µl Taq Polymerase (5u/µl). The amplification program was 4 minutes preheating and initial denaturation at 94^{o}C, then 39 x 15 seconds at 94^{o}C, 1 minute primer annealing at (45^{o}C/ 48^{o}C) based on primers used, 1.30 minutes extension at 72^{o}C. The final extension for 7 minutes at 72^{o}C was followed. The PCR reactions were stored at 4^{o}C until loading on gel for electrophoresis.

An agarose gel (1.67% agarose with 100 ml 1xTBE) prepared and 8µl amplification product of each sample with 2µl loading dye was loaded on gel. DNA seizer or DNA marker 500 bp was used to estimate molecular weight. The electrophorese were done for 2 hours at constant voltage of 100V. Gel picture was taken after staining with ethidium bromide (10mg/ml) which was mixed with 450 ml distilled water for 30 minutes and destained for 30 minutes.

4.7. Data scoring and Analysis

Each fragments that was amplified using ISSR primers, was treated as a unit character and scored as '0' for absence, '1' for presence and '?' for missing data. Based on recorded bands different software's were used for analysis. POPGENE version1.32 software (Yin *et al.*, 1999) was used to calculate genetic diversity for each population as number of polymorphic loci and percent polymorphism. Analysis of molecular variance (AMOVA) (Excoffier *et al.*, 2006) was used to calculate variation among and within population using Areliquin version 3.01 (Excoffier *et al.,*2006).

Shannon–Weaver diversity index (H') was calculated as $H' = -\sum pilog2pi$ where pi is the frequency of a given band for each population (Lewontin, 1972). Shannon-weaver index of diversity was used to measure the total diversity (Hsp) as well as the mean intra –population (Hpop). The proportion of diversity between populations was then calculated as (Hsp-Hpop/Hsp).

NTSYS- pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek *et al.,* 1999) software were used to calculate Jaccard's similarity coefficient, which is calculated with the formula:-

$$S_{ij} = \frac{a}{a+b+c}$$

Where,

'a ' is the total number of bands shared between individuals i and j,

'b' is the total number of bands present in individual i but not in individual j and

c is the total number of bands present in individual j but not in individual i.

The unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973) was used to analyze and compare the individual genotypes and generates phenogram using NTSYS- pc version 2.02 (Rohlf, 2000). The neighbor joining (NJ) method (Saitou and Nei1987; Studier and Keppler, 1988) was used to compare individual genotypes and evaluate patterns of genotype clustering using Free Tree 0.9.1.50 Software (Pavlicek *et al.,* 1999). The major difference between the two algorithms is that UPGMA assumes equal rates of evolution (molecular clock assumption) along all branches, whereas neighbor joining assume variations in the rate of change (Saitou and Nei, 1987; Studier and Keppler, 1988; Lan and Reeves 2002).

To further examine the patterns of variation among individual samples, a principal coordinated analysis (PCO) was performed based on Jaccard's coefficient (Jaccard, 1908). The calculation of Jaccard's coefficient was made with PAST soft ware version 1.18 (Hammer *et al.,* 2001). The first three axes were later used to plot with STATISTICA version 6.0 software (Hammer *et al.,* 2001; Statistica Soft, Inc.2001).

5. Results

5.1. Banding patterns and ISSR primers

Out of the six primers tested initially, three gave relatively clear banding patterns and were selected and used in this study (Table 5.1). The molecular weight of the bands amplified were in the range of 400 bp to 3.5kb. A total of 29 bands were scored from the three primers. Ten bands were scored from 811-H and the lowest were scored from 827-H (6). Of all the primers, higher number of bands (13) was scored from primer 818_H.

Table 5.1 Fingerprint patterns generated using six ISSR primers.

Primers used	**Repeat motif**	**Amplification pattern**	**Amplification of specific bands**	Number of scorable bands
811- H	$(GA)_8C$	Good	Good with smear	10
818- H	$(CA)_8G$	Good	Good	13
827- H	$(TC)_8C$	Good with smears	Good with smears	6
826- H	$(GACG)_4$	Only some samples are amplified	-	-
809- H	$(GA)_8G$	Only one sample amplified (further optimization required)	-	
816- H	**$(CA)_8A$**	**smears**	**smears**	-

5.2. ISSR band specificity of *Rana* populations

As shown in Table 5.2, allels of primer 818-8 and 811-3 respectively, were found to be specific fragments for the Kotebe population, Locus 4 of primer 818 and locus 1 of primer 827 were found specific for the Entoto population, Locus 5 of primer 873 was found to be specific for the Gulele population and locus 13 of primer 818 and locus 1 of primer 811 were found to be specific for the Akaki *Rana* population (Table 5.2).

Table 5.2 List of specific bands for *Rana angolensis* populations studied

Population	**Locus**
Kotebe	818-8, 811-3
Entoto	818-4, 827-1
Gulele	818-5,
Akaki	818-13, 811-1

5.3. Polymorphism

The number of polymorphic loci ranges from six for 827-H to 13 for 818-H and all primers are dinucleotide repeats. Among all the populations studied, *Rana angolensis* populations in Akaki were found to have the highest percentage polymorphism (68.97%), and the Gulele population showed the least percentage polymorphism (10.34%). Kotebe and Entoto populations showed 41.38% and 55.17% polymorphism, respectively (Table 5.3).

5.4. Gene diversity

Among *Rana* populations considered in the present study, the Akaki population has higher gene diversity (0.22) as compared to Kotebe (0.15), Entoto (0.17) and Gulele (0.03). Generally, *Rana* populations found in the upstream (Entoto) and downstream (Akaki) of the Kebena river were found to have relatively higher gene diversity than the other populations studied.

Table 5.3 Number of polymorphic loci, percent polymorphism and genetic diversity of *Rana angolensis* populations.

Population	With all primers		
	NPL	**PP (%)**	**GD**
Kotebe (r1-r10)	12	41.38	0.15
Entoto (r11-r20)	16	55.17	0.17
Gulele (r21-r30)	3	10.34	0.03
Akaki (r31-r4o)	20	68.97	0.22
Average	12.75	43.96	0.1425

NPL= number of polymorphic loci, PP (%) = percent polymorphism, GD= gene diversity for each population and over all populations.

5.5. Shannon-Weaver diversity index

The overall analysis with the three ISSR primers indicated that *Rana angolensis* collected from downstream of Kebena River (Akaki) was found to be more diverse as compared to the other three samples. Generally, the two Kebena River populations, upstream (Entoto) and down stream (Akaki) showed more within population diversity (0.3319 and 0.6617, respectively) as compared Kotebe (0.3193) and Gulele populations (0.1120) (Table 5.4). Higher genetic diversity were observed within populations (61.66%) of *Rana* as compared to among populations (38.34%).

Table 5.4 Shannon-Weaver diversity index within and among *Rana angolensis* population.

Population	Shannon-Weaver diversity index (H')
Kotebe	0.3193
Entoto	0.3399
Gulele	0.1120
Akaki	0.6617
Hpopn	**0.3582**
Hsp	**0.5729**
Hpopn/Hsp	**0.6166**
1-Hpopn/Hsp	**0.3833**

Hpopn =mean genetic variation for population, Hsp=mean genetic variation for the entire data, Hpopn/Hsp =proportion of genetic variations within *Rana* populations and 1-Hpopn/Hsp = proportion of genetic variations between *Rana* populations.

5.6. Analysis of Molecular Variance (AMOVA)

Analysis of molecular variance revealed that higher percentage of variation is attributed to variations among populations within groups (49.4%) followed by variation among groups and within populations with 26.4% and 24.2%, respectively (Table 5.5). AMOVA also revealed that variations among populations within groups, variation among groups and within populations were highly significant (P=0.05).

Table 5.5 Analysis of Molecular Variance (AMOVA) of *Rana angolensis* populations in Ethiopia.

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation Indices	P
Among Populations	3	30.100	0.79194 Va	27.25	0.27	0.05
Within Populations	36	76.100	2.11389 Vb	72.75		0.05
Total	39	106.200	2.905			

Population specific FST indices

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Pop#	Name	FST
1	"kotebe"	0.25657
2	"entoto"	0.28181
3	"gulele"	0.32578
4	"akaki"	0.22598

5 .7 Genetic Similarity

Jaccard's coefficients of similarity indicated (Table 5.6) (Figure 5.1) high similarity between Kotebe and Entoto *Rana* species (0.62). The least similarity was found between Entoto and Gulele (0.11). A similarity coefficient of 0.38 was observed for the Gulele and Kotebe Populations, (0.37) for Akaki and Kotebe, (0.44) for Akaki and entoto and (0.380 for Akaki and Gulele (Table 5.6).

Table 5.6 Similarity matrix for Jaccard's coefficients for four *Rana angolensis* populations based on the 29 bands obtained with 3 ISSR primers.

Population	Kotebe	Entoto	Gulele	Akaki
Kotebe	1.00			
Entoto	0.62	1.00		
Gulele	0.38	0.11	1.00	
Akaki	0.37	0.44	0.38	1.00

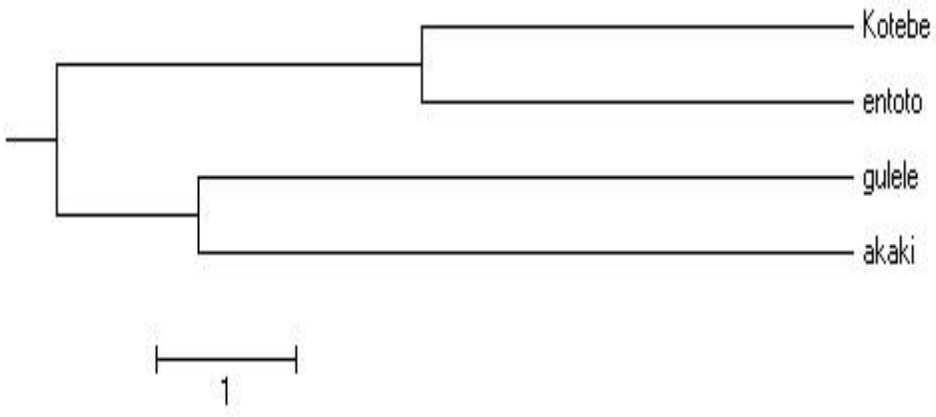

Figure 5.1. UPGMA based dendrogram for four *Rana angolensis* populations using 3 ISSR primers.

5.8. Clustering Analysis

UPGMA and neighbour joining analyses were used to construct dendrogram for the 40 individuals based on 29 bands obtained with three ISSR primers (Figure 5.2). Accordingly in both dendrograms, the four populations (Kotebe, Entoto, Gulele, Akaki) do not appear to have their own cluster separately (Figure 5.3 and 5.4). Generally, all *Rana angolensis* populations clearly observed not in separated groups in both UPGMA and neighbour joining analyses.

Rana populations in the upstream of the Kebena river collected at Entoto and population at downstream collected at Akaki were expected to form a cluster based on location proximity and movement. Unlike those expectations, both populations failed to show a clear cluster.

Figure 5.2. Dendrogram for 40 individuals of 4 *Rana angolensis* populations obtained using the unweighted pair group method with arithmetic average (UPGMA) of 29 PCR bands amplified (presence absence data) by three primers (818, 827 and 811). The UPGMA algorithm is based on Jaccard's coefficients obtained after pair wise comparison of the presence-absence fingerprint.
In the above tree, individuals from each population have intermixed patterns and are clustered together, like those from Entoto and Gulele (r18 and r30). It is shown that populations from each of the study sites could not form separate cluster.

Figure 5.3: Neighbour-joining analysis of 40 individuals based on 29 PCR bands amplified by three primers (Three samples, 41-43, were included for comparison) . The neighbor joining algorithm is based on Jaccard's coefficients obtained after pair wise comparison of the presence-absence fingerprint. One major cluster is shown to include individuals from Gulele, Akaki and Entoto. Overall, Distinctiveness of the study populations could not be revealed using Neighbour-Joining analsis.

5.9. PCO Analysis

All the data obtained using the three ISSR primers were used in PCO analysis using Jaccard's coefficients of similarity. Three Major clusters have been revealed with the two major clusters having two sub-clusters. However, the clusters do not represent a single population, and individuals from the different study sites are shown intermixed. Using two coordinates (Figure 5.4 and 5.5) almost similar result was observed like that of three coordinates.

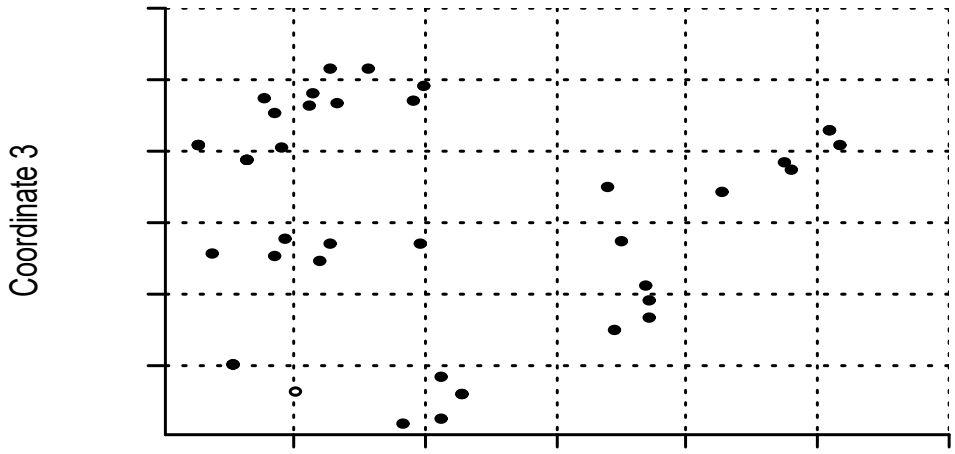

Figure 5.4 Two dimensional representations of four *Rana angolensis* populations based on Jaccard's similarity coefficients.

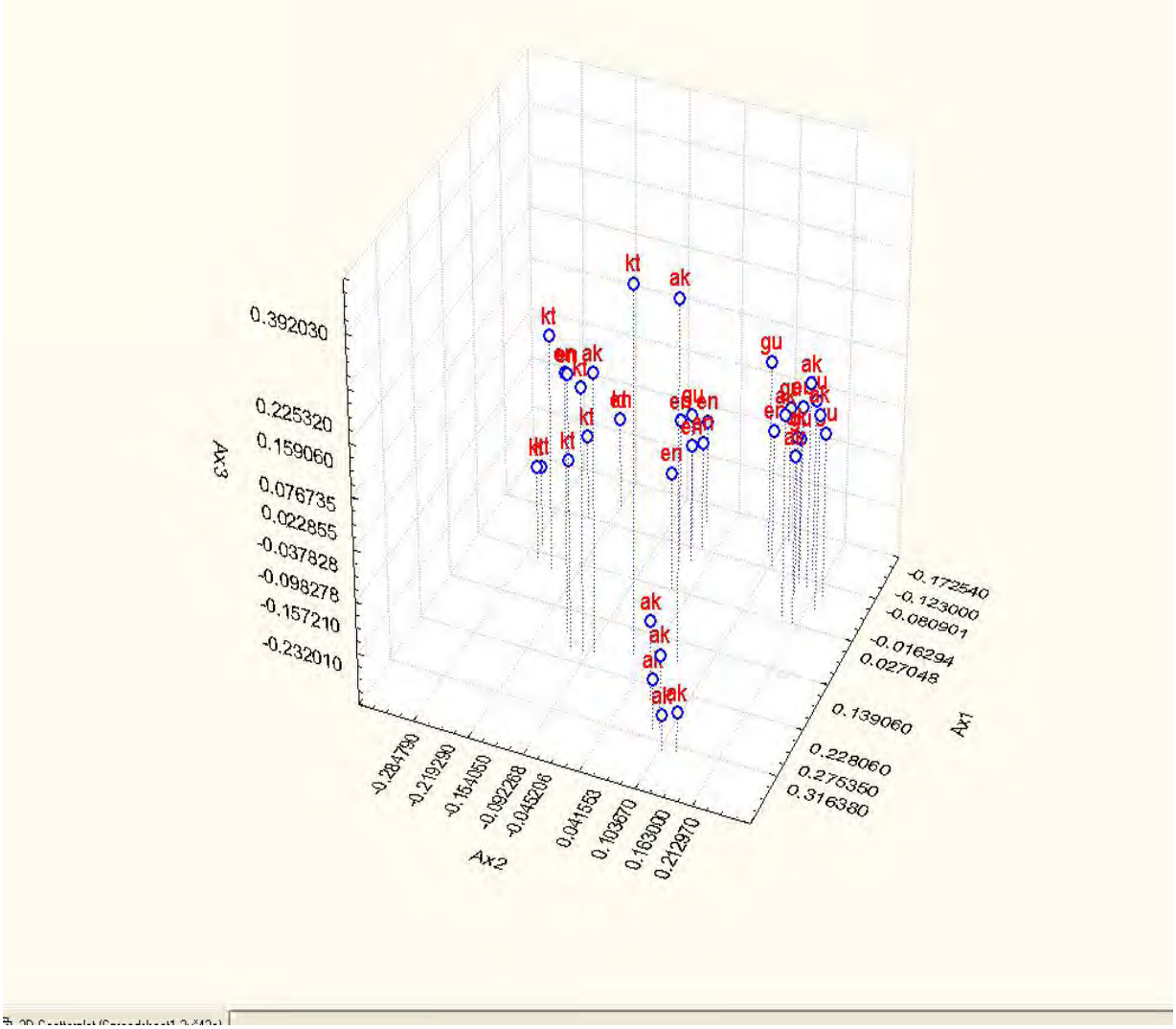

Figure 5.5 Three dimensional representation of principal coordinate analysis of genetic relationships among 40 individuals of four populations of *Rana angolensis* inferred from similarity matrix using the Jaccard's index. NB: Kt= Kotebe, Ak= Akaki, Gu= Gulele and En= Entoto)

6. Discussion

6.1. Genetic diversity of *Rana angolensis*

In recent years molecular marker systems like ISSR and RAPDs have been used for diversity analysis in amphibian species. However, most of such studies were conducted on the New World species, rather than those of Old World which comprises around 90% of the global amphibian diversity (Williams *et al*., 2001). ISSR primers have the advantage over RAPD systems due to their reproducibility and cost effectiveness. The present results confirm that ISSR marker is efficient in detecting polymorphism within and among populations *Rana angolensis* and may also be used any closely related species. Thus, ISSR marker system will provide a useful tool in the future design of collection strategies for conservation and use of amphibians.

Amphibians are known to exhibit a higher degree of population subdivision than any other major animal taxon (Parker and Keisker, 2004). The present study has revealed that genetic diversity within populations of *Rana angolensis* in the study sites is significantly greater than the differences between the populations. Several factors for the interpretation of these interesting results are considered. If there is no hibernation in this species, there is a high possibility of individual movement between populations throughout the year, especially in those dwelling upstream and down stream of the same river. Intermixing of populations through individual movement is also possible in populations living in tributary rivers that may drain into a main river. There is a high possibility of individual transfer even between populations in land separated habitats, given that amphibians could live and survive in terrestrial environments that separate those habitats. Hibernation is the main barrier of gene flow in many amphibian and reptilian species (Tyler, 1999). In other words, genetic drift under natural selection could result in a deep divergence of these species (Tyler, 1999). The present study has included both isolated habitats and river systems, with two of the sites located at upstream and downstream of the Kebena River. One population at Gulele was collected from an isolated pond which is frequently subject to environmental disturbances.

While interpreting the results in the light of the above mentioned factors (hibernation and movement), higher diversity in populations living with closer proximity to each other and or live on contiguous habitats is expected (e.g flowing rivers). This is true with regard to populations from Akaki and Entoto, which share a common river. The Akaki population has the highest percentage diversity (68.97%) while the Entoto population has the second highest (55.17%). These results are consistent with expected outcomes given that there is a high possibility of movement between individuals in these populations, and that they are active through out the year. The diversity within individuals in the Gulele population is the lowest among the studied populations (10.34%). This might be due to inbreeding and reduction of population since the collection site is isolated and severely polluted. However, these interpretations based on individual movement and absence of hibernation could be of little help in interpreting why between populations genetic diversity is lower than within population differences.

Amphibians have an external fertilization system in which embryos develop outside the mother's body. External fertilization is the main cause of interspecies hybridization in many frog species (Alexander, 2001). This could also accelerate diversity and gene flow within species by promoting cross fertilization of gametes from different individuals (Alexander, 2001). It is thought that around 75% of natural interspecies hybridizations occur due to the external fertilization system (Williams, 2001). While interpreting the results from the present study, the role of external fertilization is inevitable in the observed diversity patterns. Whereas Populations in the isolated habitat of the Gulele area are not expected to display high genetic diversity even in the presence of external reproduction, contiguous populations like Akaki and Entoto are likely to reveal higher (highest in the present study) DNA polymorphism pattern. One could consider the correlation between the breeding season and frequency of individual movement. Most frog species are highly active during the breeding season. Many *Rana* species breed during the rainy season when there is a relatively favourable environment for the development of embryos. In addition, there will be higher incidence of movement between populations during the breeding season especially in those living in rivers due to the increase in the volume of water facilitating a stronger flow.

This could be one possible scenario in populations of Akaki and Entoto, where populations in the upstream (Entoto) will be able to coalesce with downstream populations (Akaki) during the breeding season. In the light of such circumstances, it is perceptible that there will be higher chances of cross fertilization between individuals of these populations. Such incidences could therefore contribute to the observed diversity patterns. There is a possibility of movement of gametes between adjacent populations. In most frog species including *Rana angolensis*, eggs are laid in the water and they develop independently, and larvae could live whereever there is a favourable environment. This could enhance chances of gene flow and hybridization even in the absence of individual movements as unfertilized eggs from one population could easily be drawn into another locality. In the present study, such circumstances could be inevitable in interpretation of the observed results.

The external fertilization system in amphibians makes them particularly sensitive to environmental disturbances. Such reproductive behaviour may make many amphibian populations susceptible to potent mutagenic pollutants, like industrial wastage found in many rivers (Tajima, 1989). Given the permeability of the amphibian egg to many macro molecules, it is perceived that incorporation of any mutagenic material into the gametes could result in many kinds of mutations in the primary genomic sequence. One possible scenario in such cases will be that point mutations could occur some of the critical gene sequences, like in any of the components of the DNA repair machinery, which will then accelerate the accumulation of genome wide uncorrected mutations. However, to date, little research has been done on the role of environmental pollution in genetic diversity of amphibian species. In present study, it is unclear whether the level of environmental pollution in the study sites is significant enough to have an effect. Besides, it is not clear as to what pollutants are found in each of these sites. Therefore, it will be premature to conclude on the role environmental pollution in the population genetics of the species in the study.

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Overall, *Rana angolensis* population in Akaki was observed to show higher percent polymorphism and Shannon-Weaver index as compared to other samples studied here. This

could be due to either of the above mentioned factors, while emphasize on absence of hibernation and the high rate of admixture with upstream *Rana* populations. The results from the Gulele populations clearly marked the role of isolation in minimizing genetic diversity and polymorphism between individuals by promoting inbreeding.

6.2. Distinction of *Rana* populations

All the clustering analysis (UPGMA, NJ and PCOs) have shown that *Rana angolensis* populations considered in the present study are not distinct from each other. Zhang *et al.* (2003) with RAPD marker observed distinct group of the *Rana castebiena* populations in northern USA and placed apart on a UPGMA cluster each study populations, which were isolated from each other by a long tract of terrestrial environment. However, those results could not be reproduced using ISSR primers on the same set of populations (Zhang *et al*. 2003). In the present study, while other marker systems could reveal distinct populations using the same study sites. However, clustering analysis could not clearly place separate groups. Increasing Sample size and the number of primers could reveal different clustering results. Taking into account the above mentioned factors (movement and year round activity), it was predictable that populations in Gulele would form separate cluster, while populations in Akaki and Entoto would be distinct from the other populations by grouping around a single cluster. To the contrary of what we anticipated, UPGMA based dendrogram clustered Kotebe and Entoto while grouping Gulele populations with those of Akaki. However, there is no clear clustering pattern observed by PCO or NJ.

The introgression of one population into another could nullify the genetic isolation of the participating populations. In contrast, geographical isolation could accelerate a genetic isolation or even speciation. With the same reasoning, one could expect the isolated population of Gulele to have its own cluster and it will be logical to group Entoto and Akaki together. It will be appropriate to draw conclusions on distinctiveness of the populations studied in this work after a test using other marker systems. This shall be reasonable, as there are evidences that underscore the differences between different marker systems in generating clustering data. Another possibly useful classification would be that of morphological data as different habitats could exert

selection pressure in the living populations and favouring those with highly adapted morphology and genetic structure.

6.3. Genetic differentiation and population structure

Analysis of molecular variance (AMOVA) showed that of the total variation, 27.25% attributable to among populations within groups and 72.75% to within populations. This is further supported with partitioning genetic diversity analysis of Shannon's and jaccards similarity coefficients. The UPGMA and neighbour joining tree do not show a clear grouping and differentiation based on populations of origin. Thus, it has not been confirmed that the different populations that are used in this study are distinct. This is the first analysis of molecular diversity in urban dwelling *Rana angolensis* species in Ethiopia. However, there should be further analysis with additional marker systems, or using more primers and samples (with ISSR) in order to mark distinct populations according to their area of origin.

7. Conclusions

Ananlysis of Molecular Variance (AMOVA) and shannon-Weaver diversity index have revealed that genetic diversity within population is greater than that of among populations. *Rana angolensis* populations in Akaki have shown the highest diversity among all populations (68.97%) while populations in an isolated and non-flowing water body at Gulele has the least diversity (10.34%). All the clustering analysis (UPGMA, NJ and PCOs) in this result have not clearly shown that each populations is distinct from each other. Genetic similarity test using the Jaccard's coefficient of similarity has revealed that high similarity was observed between Kotebe and Entoto *Rana* species (0.62). The least similarity was found between Entoto and Gulele (0.11). A similarity coefficient of 0.38 was observed for the Gulele and Kotebe Populations, 0.37 for Akaki and Kotebe, 0.44 for Akaki and Entoto and 0.38 for Akaki and Gulele.

In conclusion, through ISSR analysis, all the diversity parameters confirm that there is higher diversity in some populations (Akaki and Entoto) and small variation within populations of Guele. Furthermore, the Akaki *Rana angolensis* population is found to be diverse as compared to other populations studied.

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8. Recommendations and research needs

- This study is not exhaustive in terms of sample size and area coverage. Hence, more survey and sample collection are to be carried out so as to cover other areas where *Rana* populations could be found.

- Generally, genetic diversity of amphibian populations is expected to be eroded as a result of reduction of population and smaller effective population size. Furthermore, habitats are threatened by the expansion of agricultural activities and overgrazing. In urban areas, expansion of settlements and environmental pollution clearly affect the resident amphibian populations. Thus, it would be appropriate to study geographical and ecological status of the populations prior to initiation of population genetic studies.

- Molecular phylogenetics and cytological analysis of *Rana* species (and other amphibian species as well) in Ethiopia should be carried out to work out the distribution, phylogeny and their evolutionary relationships.

- Analysis with codominant markers system, like microsatellites, needs to be conducted to better understand and estimate the gene flow and levels of inbreeding.

- Further study is needed with regard to the level of environmental degradation, level of pollution and habitat fragmentation in urban river systems.

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