THE RELATIONSHIP BETWEEN GENETIC AND SHAPE VARIATION IN ENDEMIC AND ENDANGERED FRESHWATER FISH SPECIES *PSEUDOPHOXINUS*

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

THE RELATIONSHIP BETWEEN GENETIC AND SHAPE VARIATION IN ENDEMIC AND ENDANGERED FRESHWATER FISH SPECIES *PSEUDOPHOXINUS*

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Evolutionary models addressing interaction between genetics and morphology propose that during development, morphological traits of organisms are under canalization selection resulting in constancy in morphology through evolutionary time. The hypothesis of genetic homeostasis predict that because of developmental buffering effects of heterosis, high level heterozygosity results in low level of morphological variance from the norms of canalized shape of the population. The aim of the present study is to test whether the variation in shape of organisms is negatively correlated with genetic variation in *Pseudophoxinus* populations. Sample collection was performed from eight localities for four different *Pseudophoxinus* species (*P. crassus, P. battalgili, P. egridiri, P. sp*) in Central and South Anatolia in summer period of 2006. Shape variation of the specimens was determined using geometric morphometric methods. Genetic variation was based on six microsatellite and ten allozyme loci. All the microsatellite loci were found to be polymorphic. However, the percentage of monomorphic locus for allozymes varied from 90% to 60% per population. Statistically significant negative correlation was observed between shape and genetic variation derived from microsatellite data. However, this was not the case for allozyme heterozygosity; there wasn't any significant relationship between shape variation and

allozymes heterozygosity. Low number of polymorphic loci observed in allozymes may prevent to reveal possible relationship between shape and genetic variations. As a result, the present study confirmed the hypothesis of genetic homeostasis for microsatellite data.

Keywords: Population Genetics, Geometric Morphometry, *Pseudophoxinus*, Allozymes, Microsatellites.

ÖZ

ENDEMİK VE SOYU TEHLİKE ALTINDA OLAN İÇSU BALIK TÜRLERİNDEN PSEUDOPHOXİNUS' DA ŞEKİL VE GENETİK ÇEŞİTLİLİK İLİŞKİSİ

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Genetik ve morfolojik karakterlerin ilişkisini konu alan evrimsel modeler, organizmaların gelişimsel süreçlerinde belirgin bir morfolojiye kanalize olmalarını sağlayacak seçilime uğradıkları ve bu durumun evrimsel süreçte morfolojik özelliklerde bir kararlılığa sebep olduğu öngörülmektedir. Genetik homeostasis hipotezi, heterozigotluğun gelişimsel süreçteki tampon özelliğinden dolayı, toplumların sahip oldukları yüksek heterozigotluk seviyelerinin toplum içindeki bireylerin kanalize olmuş morfolojiden gösterekleri çeşitliliğin azaltacağını önermektedir. Yapılan çalışmanın amacı, iç su balık guruplarından *Pseudophoxinus* (Cyprinidae) toplumlarında şekil çeşitliliğinin genetik çeşitlilikle ters yönde bir karşılıklı ilinti gösterip göstermediğini araştırarak genetik homeostasis hipotezini test etmektir. Dört farklı *Pseudophoxinus* türü (*P. crassus*, *P. batalgili*, *P. egridiri* and *P. sp.*) iç ve güney Anadolu göl ve akarsularından sekiz farklı bölgeden 2006 yaz döneminde örneklenmiştir. Örneklerin şekil çeşitliliği geometrik morfometri yöntemi kullanılarak belirlenmiştir. Genetik çeşitlilik hesaplamaları altı mikrosatelit ve on alozim belirteci üzerinden yapılmıştır. Tüm mikrosatelit belirteçleri polimorfik bir yapı göstermektedir. Ancak, alozimlerdeki monomorfik belirteç oranları toplum başına %90 ila %60 arasında bulunmaktadır. Mikrosatelit verilerinden elde edilen heterozigotluk seviyesiyle sekil farklılığı arasında

istatistiksel olarak anlamlı ters yönlü bir ilişki bulunmuştur. Ancak alozimlerle şekil çeşitliliği arasında istatistiksel olarak anlamlı herhangi bir ilişki bulunamamıştır. Alozimlerde gözlemlenen düşük seviyelerdeki polimorfik belirteç oranının, şekil çeşitliliği ile arasında olabilecek herhangi bir ilintinin açığa cıkamasında engel oluşturmuş olabilir. Sonuç olarak, genetik homeostasis hipotezi yapılan bu çalışmada mikrosatelit verilerince doğrulanmıştır.

Anahtar Kelimeler: Toplum Genetiği, Geometrik Morfometri, *Pseudophoxinus*, Alozim, Mikrosatelit.

To my mother and father

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CHAPTER 1

INTRODUCTION

About half of the species of plants and animals recently existing on Earth are expected to become extinct mainly because of human activity in less than a century (Primack 1998). Changing environmental conditions with an increasing acceleration due to the factors such as global climate change, habitat destruction, industrial pollution, and species introduction have threatened biological diversity in 20th century. The resulting loss of natural area forces species to exist in only fragmented populations of small size whose future is in doubt (Smith and Wayne 1996; Thomas *et al*. 2004).

Adaptation in changing environments depends on the genetic variation of a population in its gene pool (Toro and Caballero 2005). The spatial and temporal genetic variation in a population allows them to be able to change or evolve in response to changing environmental conditions (Moran 2002). Especially after development of marker-based genetics methods in the last decades, a large number of studies have reported that, there is a positive correlation between heterozygosity and fitness-related traits for variety of organisms (David 1998). It has been well known phenomenon since the beginning of the 20th century by geneticists that increasing heterosis in inbreed lines of organisms could dramatically affect the fitness component such as growth, survival, fecundity (Mitton and Grand 1984).

Fragmented and small population size with low gene flow would have been in genetic drift that will cause a reduction in genetic diversity. Genetic drift may lead to random fixation in these populations. If some of the alleles that have become fixed are deleterious recessives, the average fitness of individuals will be reduced. Lynch and

Gabriel (1990) put forward the idea that random fixation of deleterious recessives alleles resulted from genetic drift may cause extinction of the fragmented and small threatened populations. A reduction in fitness due to the genetic drift known as inbreeding depression may also lead to a reduction in population size. The continued decline in population size may increase the speed and proportion of genetic drift which further decrease population size. This synergetic interaction between inbreeding depression and population size drags the population in an "extinction vortex" (Mills *et al*. 1993; Soule and Mills 1998). Conservation genetics studies on threatened populations show that when gene flow is maintained between fragmented and small populations, genetic diversity can be increased. In the studies of Westemeier *et al*. (1998), improvement of gene flow between fragmented small prairie chicken populations is the best example of conservation action mitigating the risk of extinction.

Conservation and managing of endangered populations have been supported in many ways by employing molecular markers such as microsatellite, allozymes, and DNA sequences (Caballero and Toro 2002). These markers are widely used to describe patterns of genetic diversity as both within and among lineages and also to infer the evolutionary interaction between species at the ecosystem level. Furthermore, the information obtained from molecular markers can be combined with ecological information and other biological data such as morphology and thus can provide very useful information for management and conservation of endangered species (Moran 2002).

1.1 The Relationship Between Morphology and Genetics

The shape of organisms has received big scientific interest in the last centuries because shape is the major component of an organism's phenotype (Ricklefs and Miles 1994). Characteristic of phenotypic traits of a population formed by genetic background and environmental conditions are concerned with evolutionary perspective and used for phylogenetic diversification of populations (Adams *et al*. 2007). Morphology have also

direct link with some of the fitness traits such us feeding efficiency, locomotor performance, vulnerability to predator and reproductive success (Ricklefs and Miles 1994).

Although little is known about the inheritance of morphological characters, it is commonly assumed that phenotypic variation is closely related with genetic variation in the evolutionary theory (Boag 1983). Studies showed that natural populations have wide range of genetic variation that determines the range of phenotypic variation. The existence of that phenotypic variation in a natural population is then shaped by pressure of natural selection (Clarke 1979). Then the direction and level of the natural selection on the phenotypic traits form characteristics of morphology of the populations (Leary *et al*. 1985; Swain 1991; Corti *et al*. 1988).

Several models have been developed to express how population develops a characteristic morphology of a species despite abundant environmental and genetic variations. Waddington (1942) developed the "*canalization of the phenotype*" one of the earliest and important theories addressing the interaction between genetic and morphological traits in an evolutionary perspective. He proposed that, genetic basis of phenotypic variation have been under selection of canalization during development. Individuals having variation from the canalized shape should have been eliminated by natural selection. That process result in a relative constancy in phenotype of the species through the evolutionary time (Waddington 1942; Lener 1954; Mukai and Nagano 1983; Boag 1983; Leary *et al*. 1985).

An additional theory called "*genetic homeostasis*" have been developed by Lerner (1954) associated with the relationship between genetic variation and developmental homeostasis under the light of Waddington theory (1942). The level of heterozygosity is concerned with various fitness-related morphological traits for a variety of organisms and it will increase the ability of individuals' resistance against environmental and genetic variability during the development. So, genetic homeostasis theory proposed that more heterozygote individuals should have more developmental stability than

homozygote individuals in a population. Developmental homeostasis refers to the ability of an organism to maintain a stable development that will produce the fittest phenotype. Lerner (1954) predicted that there should be a negative correlation between morphological variation and level of heterozygosity. High level heterozygosity results in low level of morphological variance from the norms of canalized shape of the population due to developmental buffering effects of heterosis. Thus, individuals having more heterozygosity in a population would have the nearest canalized phenotypic norm of the population (Lerner 1954; Mitton and Grant 1984; Mitton and Koehn 1985; Shikanoa *et al*. 2005).

However, the results of previous studies about this subject in the literature have showed variable pattern from nonexisting to moderately and significantly positive and negative correlations. So, the pattern between genetic and phenotypic variability within and among populations is likely to be complex and by no means clear yet (Zink *et al*. 1985).

Aulstad and Kittelsen (1971) and Kincaid (1976-a and 1976-b) found that high inbreeding coefficient resulted in fry deformities in fish farm populations of rainbow trout. Studies of Leary *et al*. (1983) and Leary (1985) showed that individuals having greater heterozygosity screened at around 40 allozyme loci have higher developmental stability in morphological traits of salmonid fishes. They found that phenotypically extreme and asymmetric individuals were homozygotes. Shikanoa *et al*. (2005) found that there is strong negative correlation between mean heterozygosity at microsatellite and allozymes loci and level of vertebral deformity in laboratory cultured guppy strains. Mitton and Koehn (1985) also found negative correlation between allozymes heterozygosity and shape variability in blue mussel.

However, several studies have failed to find any tendency between heterozygosity and morphological variation. In the study of Baranyi *et al*. (1997), roach (Cyprinidae) population from Danube River showed significant shape variation involved body dept and fin size that cannot be explained by adaptive ecomorphological interpretations. However, he didn't found a significant relationship between genetic and morphological

variation due to narrow range of heterozygosity in allozymes loci. Gjerde *et al*. (2005) detected shortened tail and deformed anterior and posterior fin in cultured Atlantic salmon offspring. However, he found a low negative correlation between inbreeding coefficient derived from allozyme data and the deformity of the individuals. In the study of Zink *et al*. (1985), there was no significant relationship detected between heterozygosity at allozymes loci and morphological traits in fox sparrow and pocket gopher populations. Moreover, several studies performed on insects have failed to find any correlation between genetic and morphological variations (Fowler and Whitelock 1994; Gilligan *et al*. 2000; Carchini *et al*., 2001; Hosken *et al*., 2000).

In contrast to most previous studies, in the study of Strauss (1989), a surprisingly strong positive correlation between mean heterozygosity at 20 allozymes loci and size-independent morphological variance was discovered among 32 samples, representing 8 nominal taxa, of eastern North American freshwater sculpins (*Cottus*). That taxon is small bottom dwelling fishes of marine ancestry that have secondarily invaded Holarctic freshwaters and in North America and Asia have undergo significant Pliocence-Pleistocence radiation. They are characterized by patchy, mostly headwater distributions, low vagility, and highly variable morphology (Strauss 1989).

The next study of Strauss (1991) have been addressed several important questions that whether all region of the body are equally variable among individuals, populations and species and whether all the region of the body are equally associated with among sample patterns of heterozygosity. The results showed that cottus individuals showed larger shape variability in trunk and tail regions of their body than head and mouth. It is proposed that morphological integration is reduced in the regions of trunk and tail compared to that of the highly integrated cranial and suspensorial structures of the head. The other finding in this study is that head and mount regions are more correlated with heterozygosity than other body regions. He concluded that the least variable mensural characters are the most strongly correlated with heterozygosity. Such body regions tend to be the most discriminatory characters between close species and are responsive to disruptive selection over evolutionary time (Strauss 1991).

1.2 Morphometry

Morphometrics addressing the shape comparisons of organisms has always been scientific interest for biologist. Several shape analyses methods have been developed to reveal shape differences of organisms, produced by a variety of environmental and biological process such as adaptation, ontogenetic development, disease and evolutionary diversification (Rohlf and Marcus 1993; Ricklefs and Miles 1994; Richtsmeier 2002). However, scientists were not able to develop a general theory of measurements to obtain data within the traditional morphometric until the last decade. For example, there was no general theory of shape and an analytic method specified to characteristics of shape data. Each study has its own measurements theory and approach (Zelditch *et al.*, 2004).

1.2.1 Multivariate (Traditional) Morphometrics

Multivariate or traditional morphometriy has been used since 1960's in order to describe morphometric variation within and among groups. This technique has been concerned on using multivariate statistical analyses to set measured distances on the organisms such as linear distance measurements, length, width, height, or ratio and angles between landmarks (Marcus 1990). These variables are generally analyzed using Principle Components Analysis (PCA), factor analysis, Canonical Variates Analysis (CVA), and discriminate function analysis (Rohlf and Marcus 1993).

However such measurements have several limitations to reveal shape differences of organisms (Monteiro 2002). Firstly, generated data sets depend on distance measurement from a single point on the organism, so values cannot be independent and include errors associated with that point. Also, some of these measurements can be overlapped and concentrated on a single point, e.g several distance measurements along the body axis cross the single point of organisms, whereas, there may be no values from other parts of organism. Thus, that situation may complicate analyzing the shape

differences on overall body of organisms (Zelditch 1995). Another difficulty is obtaining the same linear distance measurements resulted from completely different shapes. For example; maximum length and maximum width or their ratios from different shapes (e.g. an oval and a teardrop) could be similar even if they are completely different organisms and shape. The source of this confusion is that multivariate morphometrics uses only the distance data between two locations but the locations of where the distances were made relative to one another was not included in the data. Furthermore, this approach produces a list of numbers from the angles, ratio or linear measurements. Thus this method cannot provide a graphical representation of shape as such measurements are not enough to capture original geometry of the studied objects (Adams, *et al*. 2004).

Allometry (changes in shape with a change in size) is the most important aspect in multivariate analyses as variables used in this method listed above is highly correlated with size. Separating shape from size can be very problematic, and the definition of size and shape are still not clearly characterized in traditional morphometrics. So developing methods for size correction is very crucial to measure size free shape variation. (Jolicoeur 1963; Sundberg 1989; Jungers *et al*. 1995). However several difficulties have been aroused in allometric measurements. For instance, many size correction methods were developed and there was a debate on which method should be used because different methods give different results (Adams, *et al*. 2004).

1.2.2 Geometric Morphometrics

In 1993 Rohlf and Marcus published a review titled ‘a revolution in morphometrics’ that was evaluated in the literature as a revolution in morphometrics. In the last decade geometric morphometrics has been used especially in fish research (Corti and Crosetti 1996; Corti *et* al. 1996; Loy *et al*. 1996; Loy *et al*. 1999-a and 1999-b; Loy *et al*. 2000-a and 2000-b; Douglas *et* al. 2001; Gallo *et al*. 2002; Cavalcanti 2004). This was a new method mostly eliminating the disadvantages originated from traditional techniques and

that has allowed scientists to measure size and shape components of organisms separately. It is based on the 2 (*x, y*) or 3 (*x, y, z*) dimensional coordinates of biologically definable homolog landmarks data from the geometry of the morphological structure. 'A landmark is defined as a point of correspondence on each object that matches between and within populations' (Zelditch *et al.*, 2004). Landmarks should be chosen on the specimens according to some criteria: a - They should be chosen among homolog anatomical point of organisms. b - Topological position of landmarks should not show big changes from one specimen to another. c - They should provide adequate coverage of the morphology. d – They can be identified repeatedly that means landmarks can be digitized correctly each time on the organisms. e – All landmarks should be localized on the same plane (not necessarily for 3 dimenational landmark coordinate systems) (Zelditch *et al.*, 2004).

However, the coordinates of the landmark data on the image of specimens would not be appropriate to use directly in shape analyses as they involve shape and non-shape variation due to different rotation, translation and scale of specimens. Indeed the concept of shape underlying the geometric morphometrics is precisely defined by Kendal (1977) that is 'all geometric information that remains when location, scale and rotation effects are filtered out from an object'. Rotation refers to variation in the orientation of the specimen characterized as movement around the axis. The relative locations of the points representing any single specimen remain the same, but the exact coordinates of these landmarks change. Translation is simply a variation in the position of the specimen on the digitizing table. It refers to a form sliding in any direction while remaining stable in terms of rotations around axes. Scale refers to variation in size of organisms. Although these non-shape variations do not alter the shape of organisms, it may not be mathematically useful to calculate configuration of landmarks. So they must be mathematically removed prior to the analyses so that shape differences can be analyzed independently from non-shape variation (Bookstein 1991, 1996).

1.2.2.1 Analyses of Landmark Data

Morphometric studies based on landmark data generally use Procrustes distance, or their tangent space approximations analyze to analyze shape of an object. In this method firstly, the coordinates of landmarks about specimens are represented in a configuration space including K x M matrix of cartesian coordinates of all specimens that describe a set of K landmarks in M dimensions. That is called *Configuration Space* with KxM dimension. (Bookstein 1996).

Differences including the shape and non-shape parameters between specimens in the landmarks coordinates are described by the model:

$$\mathbf{X_i = \rho_1^{-1}(X_0 + E_i)\, H_1^t + 1\tau_1}$$

Here τ is translation, ρ is scaling, H is rotation, E is shape differences (or called Procrustes distance), X_i is coordinates of landmarks of i^{th} specimen (called target form), X_0 is mean form of the landmark coordinates matrices (called consensus form). Consensus form represents average shape of individuals in the populations. If we rearrange the formula as:

$$\mathbf{\rho_1^{-1}\,(X_1 - 1\tau_1)\, H_1^t = E_i + X_i}$$

This model describes the differences between shapes as being due to scale (**ρ)**, rotation (**H)**, translation (**τ)**, and actual shape difference, E. Thus in order to analyze actual shape differences scale (**ρ)**, rotation (**H)**, and translation (**τ)** must be mathematically removed from the model. Indeed, parameters **τ, ρ** and **H** are called nuisance parameters because they encode information unrelated to shape variation. The estimates of shape variation must be independent of these parameters. Actual shape differences are represented as E, with the target to an average form in the model (Richtsmeier *et al*. 2002).

The first step is to remove translation (**τ)** effects in the landmark coordinate system. For this purpose, each configuration matrix of specimens is centered by averaging of the X and Y coordinates of the landmarks so that landmark configuration matrices of two different specimens differ only in the position of the centroid. After that, landmark configurations can be treated as a single point configuration in M dimension instead of K landmark configurations. That simplifies to translate all specimens to a distinct position by translating the centroid configurations along the x and y axes. This is the translation operation and does not alter the shape (Bookstein 1991).

The second step in the analyses of landmark data is dealing with the scale (**ρ)** of the configuration matrix. The most commonly used size measure in geometric morphometrics is *centroid size* which is the square root of the sum of the squared distances of the landmarks from the centroid (Bookstein 1986). All the centered configurations of landmarks are scaled to unit centroid size in this operation. Centroid size has not correlation between size and shape and it is not altered by changing the position of configuration matrices in translation operation. In the same manner, multiplying the configuration matrices by a constant factor increases the size by the same factor. Thus all the centroid sizes can be rescaled to be one without altering the shape (Bookstein 1996).

After these two operations the coordinates of shapes with K landmarks in M dimension can be considered as a single unit length vector corresponding to a single point in a space called *preshape space*. The term of space is defined as a collection of objects, treated as if they are points in a plane, a volume, on the surface of a sphere (e.g. Euclidean spaces, sample spaces, shape spaces, linear vector spaces, etc.). When the shape configurations have been moved from the *Configuration Space* to the *Preshape Space*, M + 1 dimension is lost by centering and scaling operations. Thus shape points in preshape space can be geometrically visualized as points lie on the surface of an M x (K-1) dimensional hypersphere with unit radius. Preshape space has 2K - 3 dimensions for 2 dimensional shapes, 3K - 4 dimensions for 3 dimensional shapes (Zelditch *et al.*, 2004).

The third step is to remove orientation (**H)** of the specimens in the configuration of aligned shapes. In order to remove orientation, a convenient orientation is selected as a references configuration. Every other target configuration is selected as the orientation corresponding to the points of closest of references configuration. This operation minimizes the distances corresponding points between target and references configurations in preshape spaces; consequently, configurations differ only in shapes. Then shape configuration is moved from preshape space to a new space known as *Shape Space* having fewer dimension as number of M (M-1)/2 dimension is lost in orientation. Consequently, shape space has 2K-4 dimensions for 2 dimensional shapes, 2K-7 for 3 dimensional shapes (Bookstein 1996). However, as the shape space is not suitable to detect the smallest distances between shape configurations, shape space is moved to a new space called as Kendall's Shape Space (Rohlf 1999).

Kendall's Shape Space has the best statistical power with the lowest mean squared error in the patterns of variations that can be detected. All the landmark configurations are represented as a single point on this 2K-4 dimensional spaces. As, Kendall's shape space and all of the spaces described above are a non-linear; non-Euclidean, positively curved shape space that landmark configurations are represented on, shape variation required special statistical techniques (Kendall 1984; Dryden and Mardia 1998; Rohlf 1999).

On the other hand, most of the multivariate statistical analyses in geometric morphometry are based on the linearization of analyses of distances and direction in a linear or Euclidean space. Consequently, as an alternative way for the shape variation analyses is that points on the Kendall's Shape Spaces is projected in a linear space that is tangent to Kendall's Shape Spaces where shape variation can be studied by linear multivariate methods. This projection likes a map that is a projection of the surface of the earth onto flat paper (Figure 1.1) (Kendall, 1984; Rohlf, 1999; Slice, 2001).

Mathematical operations explained briefly above in order to remove non-shape information (rotation, scale and translation) in the configuration of landmark data is

called as superimposition (Bookstein 1996). There are several methods such as Generalized Procrustes Superimposition (GPS), and Generalized Resistant Fit (GRF) approaches to superimpose the landmark configurations and each of them has their own protocols and criteria. GPS uses least squares criterion where the sum of the squared distances between corresponding landmarks on the two forms is minimized. GRF uses medians as superimposition parameters instead of least squares estimates. Generally it is stated in the literature that GRF is preferred when shape differences between specimens is limited to just a few landmark, on the other hand if differences between specimens is spread evenly over the objects, GPS should be used (Rohlf and Slice 1990). GPS is the most common and preferred method in this study to superimposes the original landmark configurations to a common coordinate system.

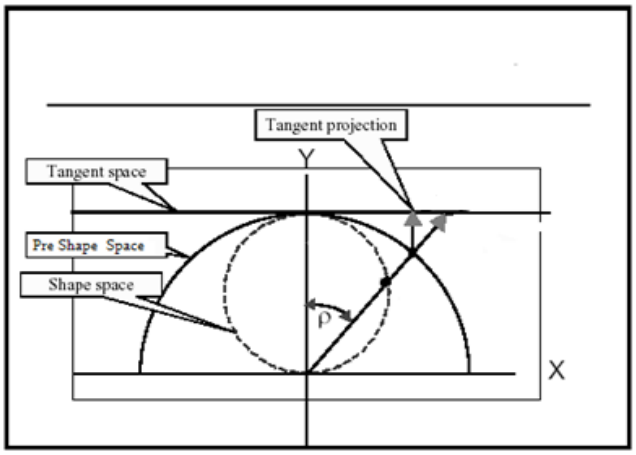

Figure 1.1: The projection of positively curved Kendall's Shape Space to a linear space called Kent Tangent Space. Shape spaces are illustrated for the simplest case for two dimensional illustration on the sheet where number of landmark is 3 and dimensions is 2x3-4=2 (Figure modified from, Rohlf, 1999).

1.2.2.2 Visualizing and Calculating the Shape Differences

Shape differences are treated as distances between corresponding points of shape configurations on the shape space that is the square root of the sum of squared

differences between the points. This quantity is called as *Procrustes Distances* (*d*) and used as metric system in shape spaces (Bookstein 1996). In order to quantify shape variation or differences between specimens, a consensus shape or a reference configuration is needed. The consensus shapes are defined as a shape having the least sum of squared Procrustes distances to the other specimens that is the average shape of the entire specimens. Shape differences are described by the differences in coordinate of landmarks in the average shape from the corresponding landmarks of the target specimens (Bookstein 1991-1996; Rohlf and Slice 1990).

Visualization for the shape differences is an important advantage in the geometric morphometry. One of the convenient methods in geometric morphometry to visualize shape variation is '*thin plate spline*' that maps the deformation in shape from one specimen to another or from average shape to a specimen (Bookstein, 1991). This method was firstly developed D'Arcy Thompson in 1917 and based on the idea of "transformation grids" where two-dimensional grid is placed over one shape, and the grid is transformed or wrapped to correspond to the morphology of the second shape. The quantity used in this method to describe shape variation for statistical interpretation is called '*partial warp scores*' that employ tangent space measure of distance (procrustes distances) as a metric (Rohlf 1993). So the result in thin plate spline is precisely the same with GPS. The advantage of thin plate spline is that it provides a visually interpretable description of shape differences differently than GPS. The change in the grid can be described as shape difference and can be clearly illustrated on a graphic of a deformation grid. The deformation grid likes an idealized thin metal plate that is constructed as the landmarks on one plate is superimposed to its homologue on the other plate (Zelditch *et al.*, 2004; Rohlf 1999; Bookstein 1996).

Shape differences between two specimens or populations can be described by two kinds of deformation grids, uniform (or known as affine) and non-uniform (non-affine). If a shape changing of a specimen occurs everywhere without disproportionate changing of one region relative to another region of an organism that situation is called uniform deformation. The partial warp scores on uniform component can be used in any

statistical procedure such as regression. Partial warp scores like the coordinates obtained by GPS that is actually, the sum of partial warp scores squares equals the squared Procrustes distances from the reference (Rohlf 1993). On the other hand, non-uniform deformation involves regionally differences on the organism that causes a bending on deformation grid. In non-uniform deformation, to map and visualize shape variation more clearly between specimens, a particular smoothness criterion such as '*minimized bending energy*' is used in this method. Minimized bending energy means that when corresponding landmarks in two specimens are mapped one specimen to another exactly, minimal amount energy is required to bend the grid to conform from one specimen (or consensus) to another. Consequently, total deformation or shape differences between specimens should be presented in combination of uniform and non-uniform component together (Bookstein 1996).

Relative Warp Analysis is a related approach of thin plate spline and has received considerable attention in the last two decades. This analysis uses the parameters of thin plate spline transformations (partial warps scores) as variables in conventional multivariate statistical analyses which are Principle Component Analyses (PCA) or Canonical Variant Analyses (CVA) …etc. In other words, Relative Warp Analysis is a PCA or CVA of the partial warps scores of thin plate spline transformations. These variables are simply weighted linear combinations of the deviations of the specimens from the consensus. Under the null hypothesis of no shape variation, the scatter of each specimen's landmark positions should deviate like digitizing error from the position in the consensus or average landmark configurations. Thus, these new variables will have multivariate normal distributions if the deviations at each landmark are normally distributed (Rohlf 1993).

Shape variation determined by geometric morphometrics can be correlated with one or more independent data such as genetic, ecological or demographic variation. Tps-Reg software was developed for multivariate multiple regression analysis between shape and independent variability (Rohlf 2003, tpsRegr, Department of Ecology and Evolution, State University of New York at Stony Brook).

1.2.2.3 Summary of Geometric Morphometry Analyses

The analysis of landmark data in geometric morphometry can be summarized in tree steps: I) GPS (Figure 2 -a and b) II) Projection of the aligned landmark coordinates on a linear tangent space for multivariate analyses III) The graphical visualization of landmarks configurations (Figur 2-c).

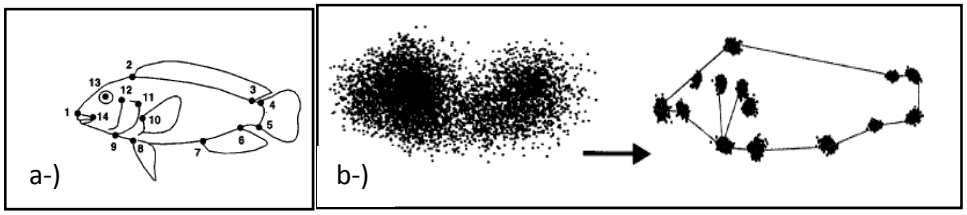

Figure 1.2: a-) Quantify raw data (landmarks recorded on body of specimen). b-) Before and after superimposition of landmarks using GPA. All landmarks from specimens were aligned according to removing of non-shape variation – (orientation, position and scale of specimens).

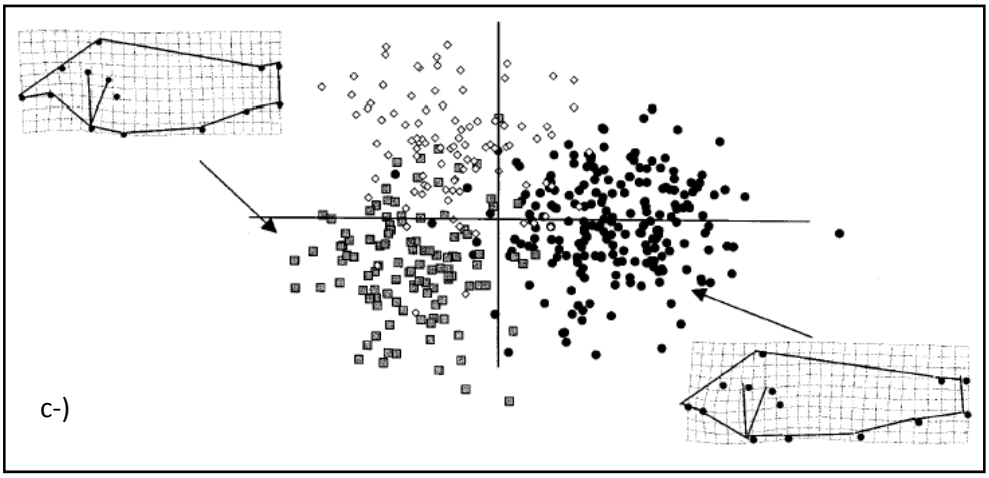

Figure 1.2: c-) Statistical analysis (CVA) and graphical presentation of results. Deformation grids for mean specimen for 2 different groups. Data and graphical representation from Rüber and Adams, 2001 (Cited in Adams 2004).

1.3 Microsatellites

Microsatellites are made up of short DNA sequences (2-6 nucleotides) that are uninterrupted tandemly repeated from about 5 to more than 100 times in the genome. They are found at many different loci in genomic DNA of every organism, including coding and non-coding regions (Goldstein and Schlötterer 2001). A number of different names have been used in the literature to describe these repeat sequence such as short sequence repeats (SSR) or short tandem repeats (STRs) that sometimes cause confusion in the literature. Microsatellite has become the most common term to describe such sequences.

Microsatellites are known as neutral genetic markers and identified by their sizes (as base pairs) of polymerase chain reaction (PCR) amplified fragments with designed primers based on flanking region sequence. However, there is no certain pattern or rule determined either about lower limit for iteration of a repetitive sequence or about how a ideal microsatellite sequence can be. Microsatellites show high abundance and high level of polymorphism owing to variation in the repeat number of uninterrupted arrays in genome. Microsatellite repeats are generally located in non-coding intergenic regions or introns and thus they are known as neutral markers. So they can be used as genetic markers in many different areas of research such as forensic applications, diagnosis and identification of human diseases, population studies, evolutionary genetics and conservation biology (Di Rienzo *et al*. 1998; Goldstein and Schlötterer. 2001; Sainudiin *et al*. 2004). Although microsatellite is described as uninterrupted tandemly repeated, most of microsatellite units amplified by PCR contain interruption or one more different repeat motif between tandem repeats that lead to difficulties in interpretation of their length variation at the population level (Goldstein and Schlötterer. 2001).

Microsatellites are considered in terms of their roles as neutral genetic markers in population genetics, however recent studies have emphasized on their functional roles as coding or regulatory elements (Ellegren 2004). Several studies showed regulatory function of microsatellites in upstream promoter regions of coding sequences that the

promoter region involving microsatellites show more enhancement activity in transcription. However, deletion constructs lacking the microsatellites tracks reduces transcription activity of the promoter. Moreover, the enhancement effects of microsatellite have also been shown in their protein binding ability that is closely related with the number of tandem repeats in a specific microsatellite tract. As coding sequence, microsatellites have been found in many proteins in which their variation in repeat number is closely related with its functional effects. Additionally, recent studies showed that microsatellite fragment variation have phenotypic effects on physiology and development of organisms, although the demonstration of this quantitative phenotypic effects associated with DNA is quite difficult. Studies on diseases in humans resulted from trinucleotide-repeat expansion showed that there is strong correlation between repeat length and age of onset and also severity of disease (Ashley and Warren 1995; Goldstein and Schlötterer 1999).

Mutations are one of the most important determinant factors of polymorphic structures of populations (Weber and Wong 1993). Rate of microsatellite mutations in *in vivo* systems is reported to be approximately 10^{-2} events per locus per replication in *E. coli*, and 10^{-4} in yeast and 10^{-3} events per locus per generation in human autosomal chromosomes. Microsatellites have their own variability leading to increased rate of mutation compared to other neutral DNA regions. (Levinson and Gutman 1987; Henderson and Peters 1992; Weber and Wong 1993).

These high rates and complexity of the mutation process in microsatellites may be influenced by several factors such as DNA slippage, mismatch repair system efficiencies in different species, length constraints, selection, point mutations, repeat numbers, repeat types, flanking regions, recombination rates, sex and age (Schlötterer 2000). Among these factors, two mechanisms can be determined as initial mechanisms that generate variability in microsatellite. The first one is based up on slippage-strand mispairing during replication and the second one is based upon that recombination could alter the length of microsatellite by unequal crossing-over or by gene conversion (Jeffreys *et al*. 1994; Goldstein and Schlötterer 1999).

Studies on yeast and *E. coli* has revealed that DNA slippage seems to be main mechanisms generating length mutations in microsatellites especially when repeat microsatellite length is more than 7 bp (Sainudiin *et al*. 2004). Slippage occurs only in single DNA double helix when nascent DNA strand mispair with the template strand in the repetitive sequence during the replication. That cause longer or shorter products of nascent strands than template strands. Microsatellites then lose or gain single or few repeats. This kind of mutation is very common types in microsatellite and has been detected in *E. coli*, yeast and also human genome. Unequal crossing-over lead to a deletion in one DNA molecule strands and insertion in the other when that occurs in long tandemly repeat sequence where recombination cannot easily determine the correct register between two strands. Thus unequal crossing-over can give rise homogenization of variants within an array of tandemly repeat sequences (Goldstein and Schlötterer 1999). Consequently, microsatellites show high variability with a few loci and it is possible to obtain unique multilocus genotypes even in individual level of a population. This property of microsatellites make difficult to discriminate populations (Estoup *et al*. 2002).

Mutation rates of microsatellite may have been affected by the length of repeat as longer repeats tend to be more polymorphic than shorter repeats, potentially because of having higher probability of misalignment configuration in longer repeats (Ellegren 2004). Moreover, purity of the repeat units may have been determined as an influence for mutation in microsatellites. Because interrupted repeats (due to insertion or base substitution) seems have less mutation rates than uninterrupted ones. There are several germline studies on human and barn swallows stated that microsatellite loci involve more gain than losses of repeat units. However the molecular mechanism leading to this kind of mutation is still unknown (Weber and Wong 1993).

1.3.1 Theoretical Mutation Models

Understanding mutation mechanisms of microsatellite that form its evolutionary process is crucial to optimize information used in many population genetics applications. The information concerning the frequency of mutations and the way of created new alleles is used for the estimation of genetic distance, divergence times, or amount of gene flow between populations. Thus much effort of microsatellites studies have focused on the development of biologically realistic mutation models. Microsatellites show inconsistency in their evolutionary dynamics of repeat array that causes difficulties in determination of which mechanisms underlying allele frequency changing in a population. There is several theoretical and empirical mutation models developed for acute estimation of population parameters in the literature (Ellegren 2004).

Classically there are two models for microsatellite mutations, Stepwise Mutation Model (SMM) and Infinite Allele Model (IAM). SMM model is the first developed to determine allozymes allele frequency but it is also applicable for microsatellites studies. In this model, mutation occurs as a loss or gain of a single repeat unit with equal probability. Thus this model can predict newly formed alleles which have already been involved in the population (Shriver *et al*. 1993; Estoup *et al*. 1995). On the other hand, IAM describe mutations that involve any number of tandem repeats and always creates a novel allele not previously involved in the population (Estoup *et al*. 1995).

SMM is often applied to microsatellite data by population geneticists because of its simplicity. It is used to define the closeness of microsatellite alleles according to their repeat length. However it is insufficient to explain all observed data. For instance, mutations have been observed to change the repeat length more than one unit in microsatellite (Huang *et al*. 2002) Also, SMM doesn't include equilibrium distribution for allele length and doesn't explain the absence of very long microsatellite alleles that are the weakness points of this model (Huang et al. 2002).

Two Phase Stepwise Mutation Model (TPM) is an alternative model developed for microsatellite mutations (Feldman *et al*. 1997). In TPM, mutation occurs in one repeat unit with probability of *p* (this corresponds to the SMM) and in more than one unit with probability of 1-*p* while the distribution of the length of multiunit mutations is geometric. TPM doesn't include upper or lower limit on the number of repeat units in an allele (Paetkau *et al*. 1995). Under the SMM and the TPM, a microsatellite is assumed to mutate at a constant rate, irrespective of its repeat length. Moreover, under these models there is no bias toward an expansion or a contraction, and thus the microsatellites are expected to grow or contract unconstrained over time.

The other alternative model to SMM is called Proportional Slippage Model (or known as Point Mutation Model) (Kruglyak *et al*. 1998). The notion of this model is that the mutation rate increases linearly with the microsatellite's length so longer microsatellites mutate more rapidly and that microsatellite undergoes point mutations (Watkins 2006).

As the mutation rate is too high, it is not easy to determine which models' output is closer to the actual rates of mutation in microsatellites (Ellegren 2004). The most straightforward method seems to be genome sequence and direct observation of allele transmission in pedigree analyses (Watkins 2006). Direct analyses of mutation rate are used to test the accuracy of mutation models. Consequently, these analyses showed that mutation rates vary with species, repeat types and loci. Testing models by direct observation studies showed that many mutations events occurred in more than one unit in microsatellites that do not support SMM model (Weber and Wong 1993, Goldstein and Schlötterer 1999). Sequencing studies revealed that flanking regions have an additional role on microsatellite variations although flanking regions are more conserved and have lower mutation rates than repeat regions (Angers and Bernatchez 1997). On the other hand, studies on human populations showed that allelic frequency distribution of many microsatellite loci consistent with TPM model (Colson and Goldstein 1999).

1.3.2. Microsatellites in Population Genetics

Although application of microsatellite data in population genetics studies is relatively recent, it has been rapidly grown and become routine over the last decade (Coltman and Slate 2003). This is because of the potential for the use of these markers in small and endangered species. Moreover, the assessment of genetic variation and its structure within and among population and possibility of unambiguous determination of parentage and precise estimation of relatedness make the microsatellite to be best candidate markers in population genetics studies (Moxon and Wills 1999).

Microsatellite can be easily obtained either by directly isolated through the isolation of species-specific markers or by application of markers isolated from related species (Schlötterer *et al*. 1991). The other advantage of the microsatellite application is that they are amplified by PCR using non-invasive sampled material (fecal material, saliva, hair … etc) that make it possible to track to population without needing direct contact with them (Goldstein and Schlötterer 1999).

However, there is some drawback of microsatellite application were reported in the literature. For example, for certain group of organisms (many plant species, several vertebrates and invertebrates, some dipterans and gastropods) it is not always easy to isolate and characterized microsatellite form from genomic DNA. Some of the PCR processing problem is another difficulty in microsatellite applications. For instance, non-amplification of certain alleles because of substitutions, insertions or deletion occurred within priming sites of microsatellite can lead to null alleles. Polymerase enzyme generated slippage products causing single base shift and size problem in the allele scoring is very common difficulty in microsatellite studies (Goldstein and Schlötterer 1999; Coltman and Slate 2003).

1.3.3 Using Microsatellites to Measure Inbreeding and Outbreeding

Microsatellite variations have been extensively used as a tool for measuring the fitness consequences of inbreeding and outbreeding of populations that are the most important parameters in conservation genetics. The fitness consequence of offspring depends on the genetic relatedness of their parents in diploid populations. It is well known literature knowledge that inbreed offspring are less fit due to inbreeding depression and outbreed offspring are more fit (heterosis) if outbreeding is not as much as in hybrid zones that may cause disruption of local adaptation leading to low fitness due to outbreeding depression (Goldstein and Schlötterer 1999). However, the process and relative importance of inbreeding and outbreeding is not clear and have been poorly documented for natural populations. There is also big debate in the literature about to the level of relatedness for inbreeding depression in natural populations. Moreover, studies showed that there is interaction between environmental condition and inbreeding depression that may not be detected in laboratory conditions. The other difficulty for natural population is that how inbreeding depression varies with the kind of trait studied that is largely untouched in natural populations and cause difficulties in making informed decisions about population managements (Goldstein and Schlötterer 1999).

There are two approaches used in the population genetics to study inbreeding and outbreeding in natural populations. The first approach is to analyze individual mean heterozygosity at sample of codominant molecular markers such as microsatellite and allozymes that is inversely correlated with inbreeding coefficient (Britten 1996). Individual heterozygosity calculated in this approach represents recent mating between relatives. A general problem in this approach is the relatively small number of polymorphic loci studied; most of the loci may be homozygotes even in the absence of inbreeding depression for a population. Microsatellites are considered as much more polymorphic markers than allozymes as they have more alleles and heterozygosity. So that, the degree of inbreeding depression can be more closely calculated by microsatellites markers than allozymes (Britten 1996).

The second approach is stepwise mutation process of microsatellites. According to this model, allele length may involve historical information about their descent. So, the measure of internal distance between individuals can be calculated which reflects the time to coalescence for the two alleles at a locus, or average across loci, the mean time to coalescence for the microsatellite studies (Weber and Wong 1993; Goldstein *et al*. 1995). In the population level, the distance squired, averaged over many loci, is linearly correlated with the time since two populations diverged. The same measure can be estimated in individual level that is donated as mean d^2. Mean d^2 can be calculated as a measure of variance in average allele length within an individual. It is concluded in this approach that intra-population variation in mean d^2 is sourced from the migration between populations causing divergent between microsatellite lengths (Goldstein and Schlötterer 1999).

1.4 Allozymes

Allozymes are codominant protein variants (alleles) that are the first major molecular genetic markers developed in 1960's (Harris 1966; Lewontin 1966). They have been widely used in systematic, evolution, ecology and conservation genetics. Although, many molecular approaches have been developed such as microsatellite, this approach is still an efficient tool in conservation genetics because of its relatively low cost and easy and rapid application procedures (Leary *et al*. 1993). There are two forms of protein generated (allozymes and isozymes) can be generated simultaneously using electrophoretic method. Allozyme is a form of an enzyme coded by different alleles at the same locus. Isozymes are also enzymes having the same function, but coded by different loci. Both forms of data are used in molecular studies in order to understand genetic variability in natural populations (Simith and Wayne 1996).

Allozyme electrophoresis technique is simply based on the separation of allelic variants (alleles) of an enzyme or other protein variants in an electrophoretic media. Migration of allozyme alleles through electrophoretic media shows differences mostly due to the

differences in their electrical charges and also due to their shape and size. Such differences in their structure and charge between alleles are considered as to be due to their amino acid composition that represents differences in the DNA sequencing encoding the protein. Thus allozymes analysis is used to observe genetic variation from the gene products (Simith and Wayne 1996). Protein samples can be obtained from any kind of tissues of studied organisms such as muscle, kidney, liver, also from nondestructive samples like blood, body mucus and fin clip (from fish), pulp of growing feathers from birds. However, as isozyme expression is often tissue specific, non-destructive sampling is not always provide data about all the examined loci (Pasteur *et al*.1988).

Proteins consist of one or more polypeptide chains (subunit) each of which is composed of amino acid series joined together by covalent bonds. Twenty amino acids common to all organisms are classified into 4 classes according to their radical groups that are non-polar radicals, non-charged with polar radical, negatively charged and positively charged. The charge of a protein is determined by the number of positively and negatively ionized amino acids in the subunits of protein. New alleles in allozymes can be created by point mutations or an insertion-deletion happened in the DNA coding sequence of the gene (Pasteur *et al*.1988).

There are 3 possibilities that may cause new alleles: In the first case, a non functional new allele resulting in low fitness probably will be eliminated by natural selection. Alternatively, if mutation can change relatively unimportant part of enzyme, new allele may be selectively neutral that may lead to genetic drift. In the third and the least possible case, an enzyme catalyzing different chemical reaction due to a mutation may be favored by natural selection and increases the fitness. Although, mutations result in amino acid composition of proteins by replacement of one amino acid by another, the net charge of protein may not be changed (Pasteur *et al*.1988). Thus, electrophoresis can only detects around one-third of amino acid substitutions. This is the most important drawback causing under-estimation of the level of actually existing polymorphism by this method (Thelen and Allendorf 2002)

Allozymes are used in conservation biology to estimate genetic variation in the matter of allozyme diversity including multiple locus heterozygosity, proportion of polymorphic loci, and average number of alleles per polymorphic locus. Low allozyme diversity is considered as past or current population bottlenecks on genetic diversity (Wayne *et al*. 1991; Hartl and Pucek 1994). Allozyme electrophoresis has been used to address many questions in conservation genetics such as population viability, estimation of effective population size (Briscoe *et al*. 1992), gene flow (Dole and Sun 1992), breeding system (Dole and Sun 1992), assessment of level of hybridization (Leary *et al*. 1993), paternity and species determination and phylogenetic systematic (Erwin 1991).

It is believed that estimated genetic diversity within population is associated with population viability. Many studies have recorded that there is strong correlation between single or multi locus allozyme heterozygosity and individual fitness (Allendorf and Leary 1986; Leberg 1990-1993). Moreover, effective population size that is very important parameter for conservation biology studies can be estimated using changes in allozyme heterozygosity and allele frequency over time (Briscoe 1992). The degree of gene flow between populations is used to assess the level of isolation for endangered populations. If the gene flow is low for a population, this population can be considered as distinct unit in management (Dole and Sum 1992). Next to the estimation of genetic diversity within population, allozymes electrophoresis is used characterized genetic relationship among population. As genetically differentiated populations have unique genetic combinations, it is suggested to be protected within the framework of special management consideration (Leary *et al*. 1993).

Hybridization is a major threat for those unique genetic stocks for some groups like freshwater fish. Present of number of unique alleles from each species in hybrid complex is used to calculate the level of hybridization. Combining the allozyme data with maternally inherited marker such as mitochondrial DNA make it possible to determine the direction of the hybrid mating between populations (Dowling and Childs 1992).

1.4.1 Limitation and Advantages of Allozyme Electrophoresis

The one of the advantages of the allozyme data is to have opportunity for comparative study for many taxa that is not possible for other molecular markers. Surveying existence data in the literature makes it possible to compare between past and present changes in genetic diversity of a population (Hartl and Pucek 1994). The low cost and easy application of allozymes electrophoresis allow rapid examination of genotypes for high number of individuals (Simith and Wayne 1996).

There are many problems reported about estimation of genetic diversity using allozymes electrophoresis methods. It has been assumed in most of the population genetics models that allele frequencies are not influenced by natural selection. However, some of the allozymes seem to be under natural selection. The problem here is that the degree of the neutrality or selection force for allozymes is unknown (Koehn *et al*. 1988). The other principle problem is sourced from sampling insufficient number of loci and individuals. It has been suggested that at least 40 loci with the large sample of individuals (e.g. a hundred of individual) should be sampled for a large population to have much confidence in heterozygosity estimates and to consider whether a loci is monomorphic. However, many of the allozyme survey do not meet these requirements. Even, large number of individuals and loci are examined, polymorphisms are often uncommon as the majority of the loci sampled within most population are monomorphic and do not provide information about genetic structure of the populations (Sjorgen and Wyoni 1994).

1.5 The Current Status of Freshwater Fish Populations in Europe and Turkey

According to IUCN reports, a third of all freshwater fish species in lakes and streams on the Earth have become extinct or endangered and around 3 to 5% of those are listed on the IUCN list of endangered fauna (Crivelli *et. al* 2000). 56% of total number of freshwater fish species in Mediterranean region are threatened (IUCN-International

Union for Conservation of Nature, http://www.iucn.org/). Because of anthropogenic effects causing habitat destruction such as are removing water for agriculture, dam constructions, industrial waste materials or acidification, fragmentation and introduction or translocation of species, natural fish populations have declined or extinct. Furthermore, nowadays, global warming has been considered as a new factor affecting on freshwater fish populations (Harrison and Stiassny 1999). Today, 131 species and 98 subspecies of fish endemic to the northern Mediterranean were identified and 31 % of these species were found in Turkey. However, the fish distribution, taxonomy and the conservation status of southern Europe and Mediterranean countries were not well studied and documented in the literature, although, this is not the case for northern Europe. Furthermore, around 10 % of freshwater fish species are listed as endangered or vulnerable in the southern Europe (Geldiay *et al*. 1998). Southern Europe especially Mediterranean basin had provided refuge areas for many species including freshwater fish populations in the post glacial expansion during the four main ice ages of the Pleistocene, so that the large number of species with large number of endemism has been found in this region (Kosswig 1955).

Cyprinidae are the richest and most important family of freshwater fish group, and its members are distributed world-wide. Cyprinid fishes of the genus *Pseudophoxinus* (Heckel 1843) is the most endangered group of Cyprinid because of the continuous damage of freshwater ecosystems in Mediterranean region (Balık 1995; Geldiay *et al*. 1998). This genus is geographically restricted and their temporal and spatial diversity in Anatolia are driven by the geological events, which shaped Anatolia and cause diversification for many other organisms (Hrbek *et al*. 2004). As environmental conditions in Mediterranean region are mostly semi arid or arid, aquatic habitats are very fragmented and isolated. That condition enhances speciation processes that result in presence of number of species within single or few hydrographic basins (Kottelat and Barbieri 2004).

1.5.1 The Biology, Distribution and Conservation Status of *Pseudophoxinus* (Heckel 1843).

Phylum: Vertebrata, Class: Actinopterygii, Subclass: Neopterygii, Order: Cypriniformes, Family: Cyprinidae, Genus: *Pseudophoxinus*.

The genus of *Pseudophoxinus* shows the distribution in temperate and subtropical regions between 42°N - 35°N latitude in Mediterranean region. The size of the *Pseudophoxinus* species varies from 6 to 20 cm depending on species. They inhabit clear waters of rivers and lakes where it forms small schools in the middle or upper layers of the water as well as close to the bottom. They frequently occur in areas of slow-moving or still water amidst stones and vegetation. In some regions they show very interesting biology since, during the dry periods of the lake it survives by sinking down into the mud and forming a slippery envelope around the body. Field observations have shown that adults feed on zooplankton, small insects, larvae and plants. They are short-lived species which occurs in spring and associated wetlands and in lowland water courses with little current. Their spawning period is ranged from April to July depending on species and region (Küçük and İkiz 2004; Özel *et al*. 2006). Temperature tolerance is between 5-20 °C. High, minimum population doubling time is less than 15 months. Most of the members of the genus are threatened due to water abstraction and habitat destruction (http://www.fishbase.org/).

The genus *Pseudophoxinus* is distributed in a wide range of Anatolian habitats with 10 endemic species (Bogustskaya 1992; Bogutskaya *et al*., 2007). Moreover, they are found in Lebanon, Syria, Israel, Jordan, Iran, Azerbaijan, Tunisia and the Balkan Peninsula. But few new species have been described both in Anatolia and Europe in last decade (Küçük and İkiz 2004; Kottelat and Barbieri 2004). Taxonomic studies of the genus *Pseudophoxinus* was started by Kosswig in 1955's and continued by several scientists like Battalgil (1944), Balık (1995), Karaman (1972), Küçük (2006). However, after the comprehensive revision of Bogustskaya (1992) and the phylogenetic study of Hrbek *et al*. (2004) there is no consensus in taxonomy of some Anatolian

Pseudophoxinus species. On the other hand, 6 species of genus; *P. antalyae* (Bogutskaya 1992), *P. handlirschi* (Pietschmann 1933), *P. battalgili* (Bogutskaya 1997), *P. crassus* (Ladiges 1960), *P. kervillei* (Pellegrin 1911), and *P. egridiri* (Karaman 1972) were identified correctly (Hrbek *et al*. 2004).

P. egridiri is endemic to Anatolia and reported as critically endangered species in International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species, 2004. Because of habitat destruction, introduction of fish predator into the inhabited lakes of these species in Anatolia, *P. egridiri* has been extirpated from Lake Eğridir which is one of the largest Anatolian freshwater lakes. Now, there is only small *P. egridiri* populations that survive in few surrounding streams (Hrbek *et al*. 2004).

P. crassus was another threatened species recorded as endangered (EN) status in IUCN Red List in 2004. *P. crassus* is an endemic species in Anatolia and distributed as fragmented populations in lakes and streams around Lake Tuz, İnsuyu, Güneşli, Haymana and Eşmekay in central Anatolia. *P. crassus* populations are threatened by overfishing and habitat destruction due to heavy agricultural activity, and water pollution (Cirvelli 2006).

P. battalgili is another endemic species for Anatolia and distributed mainly in Lake Beyşehir, Oymapınar Dam, Lake Çavuşçu and different lakes and streams in central Anatolia. Although, no data is available for demographic structure of this species, it has been believed to be declining and recorded as endangered (EN) in IUCN Red List in 2004. Natural habitat for *P. battalgili* is springs and river rather than lake itself, although it is known as lacustrine species. An introduced predator species *Sander luciopera* (Zander) is the main threat for *P. battalgili* populations (Cirvelli 2006).

P. anatolicus (Hanko 1924) is endemic for Anatolia and has distributed naturally in Lake Beyşehir. Also fragmented populations are still present in the Akgöl basin, Lake Çavuşçu, Akkaya Dam, Erğli marshes, Göyöz and İnsuyu. As *P. anatolicus* has not

natural fish predator, introduced alien predator fish species is thought to be one of the important factor for declining population size in Lake Beyşehir. Moreover, overfishing, drought and pollution are listed as the other threat for this species. *P. sp* is assessed as endangered (EN) in IUCN Red List in 2004 (Crivelli and Erk'akan 2005).

AIMS:

The aims of the present study are as follows:

- To test the hypothesis whether shape of the organism show increased variation due to level of heterozygosity in small endangered *Pseudophoxinus* populations and whether it will be possible to correlate between genetic variation and morphometric variation.

- To understand and compare population genetic structure of eight *Pseudophoxinus* populations using allozyme and microsatellite data.

- To understand and compare morphometric structure and variables of eight *Pseudophoxinus* populations using geometric morphometric data.

CHAPTER 2

MATERIAL AND METHODS

2.1. Sample Collection

Sample collection was performed from eight localities for 4 different *Pseudophoxinus* species (*P. crassus*-2 populations*, P. batalgili*- 4 populations, *P. egridiri* -1 population and *P. sp* – 1 population) in Central and South Anatolia in summer period of 2006. Sampling places, dates, number of collected specimens and their longitude and latitudes are listed in Table 2.1 and locations are illustrated in Figure 2.1. Specimens were caught using electrofishing and a net in generally around incoming river mounts in lakes and spring waters. Collected individuals were placed in hard plastic tubes individually and stored in dry ice at around - 70 °C during the field trip in order to prevent morphological deformation and genetic and molecular deterioration. After the field trip, all samples were placed in shock freezer in -80 °C in the laboratory until morphologic and molecular analyses.

Table 2.1: Species names, location names, latitudes and longitudes, dates and number of *Pseudophoxinus* individuals collected in the field.

Species names	Locations	Latitude	Longitude	Dates:	# of indv.
P.crassus	Haymana İnsuyu Village-İnsuyu River	38,7034	32,7637	08-08-06	34
	Kulu – Kozanlı Lake Gök	39,0081	32,8363	19-07-06	35
P.batalgili	Seydişehir Yalıhöyük Village-Lake Suğla	37,3041	32,0473	07-08-06	36
	Manavgat-Oymapınar Dam	36,8782	31,5200	06-08-06	33
	Ilgın-Lake Çavuşçu	38,3269	31,8642	04-08-06	37
	Seydişehir-Taşağıl River	37,3783	31,8914	07-06-06	29
P.egridiri	Eğirdir, Lake Eğridir	38,1466	30,8642	03-08-06	33
P.sp	Beyşehir, Eflatun River	37,8267	31,6746	03-08-06	24

2.2. Geometric Morphometrics

For morphometric measurements, a high resolution digital SLR camera (Nicon D70s and Nicon 60 mm macro lens) was used. Lateral body sides of specimens were photographed with the camera fitted to a tripod from a fixed distance from the fish and fish were aligned on a straight line on a table. A milimetric grid paper was used as references ruler with each fish on the table. In order to remove shading effects on the images a circular macro flash (Vivitar Macro Flash 5000) was mounted on the lens that provide sharp and clear image. Images were transferred to the computer and transformed to tps format for geometric morphometric calculation using tps-util software (Rohlf 2004). The coordinates of 9 homolog landmarks in the head region defining the biological features of the organism were used to characterize shape variation (Figure 2.2). Landmarks were digitized on lateral views of each individual

directly from the digital pictures using tps-dig2 software (Rohlf 2004). Software can be downloaded from http://life.bio.sunysb.edu/morph.

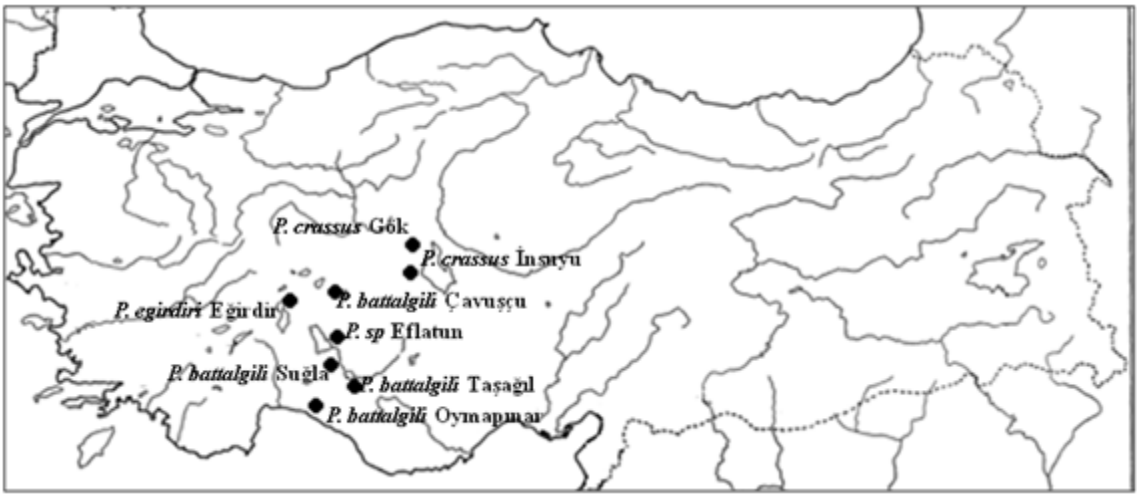

Figure 2.1: Sampling places of collected *Pseudophoxinus* populations.

The landmark configurations of each specimen were superimposed using Generalized Procrustes Analysis (GPA - or known as least-squares superimposition) in order to remove non-shape information (position, orientation and scale) from the coordinates of 9 landmarks. A mean (called as consensus) shape configuration was calculated from these superimposed data for each species and also each population. The partial warps and uniform components scores were used for the statistical analyses of shape variables within and among groups. These scores were derived from Procrustes Distance that describes shape variation of each specimen to the consensus (mean) shape of a population. Shape differences of each population from the mean shape were visualized in Cartesian Transformation Grids (CTG) and statistical significance test of the shape differences was done using pair wise test comparison. All the process was accomplished by using MORHEUS (Slice 1998) software (GPA; Rohlf and Slice 1990).

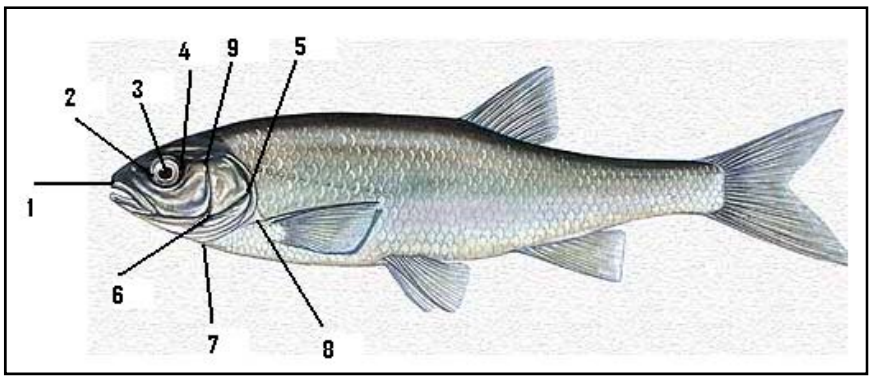

Figure 2.2: The eight landmarks used in the geometric morphometrics analysis. (1, anterior tip of upper snout; 2, and 4 circle of eye; 3, central point of eye; 5, upper right posterior edge of opercular aperture; 6, lower posterior edge of opercular aperture; 7, end of gill opening; 8, upper insertion of pectoral fin; 9, upper left posterior edge of opercular aperture. (Picture is from http://www.ittiofauna.org)

In order to describe the structure of overall shape variation among populations, partial warps and uniform component scores of each individual were combined in a data matrix and submitted to Canonical Variate Analyses (CVA) and Principle Component Analyses (PCA). Plots of PCA and CVA were drawn using IMP software (Sheets H.D., Department of Physics, Canisius College, NY-USA 2001, www.canisius.edu).

The score of centroid size of each individual were regressed with superimposed landmark coordinate data through Generalized Goodall's F-test using TpsRegr (Rohlf 1997) software in order to understand whether there is any significant variation in shape with size within and among the populations. Centroid size (the square root of the sum of the squared distances of each landmark) which is a geometric size of each individual was calculated using TpsRelw version 1.36 (Rohlf 2003). 'Procrustes Distance' values of individual that are quantification of deviation of each individual from consensus shape configuration of the population was generated using TpsRegr software (Rohlf 1997). Then, shape deviation from the consensus shape in individual level was illustrated on a digitizing grids using tps-thin-plate spline software (Bookstein, 1991).

2.3 Microsatellite Analyses

2.3.1 DNA Isolation:

Genomic DNA isolation was completed using Fermentas DNA purification kid for the specimens. Around 50 µg fish muscle was dissected using sterile dissector and transferred to sterile eppendorf tubes including 200 µl TBE buffer (Fermentas). After homogenization of the muscle tissue in the tubes, 600 µl Lysis solutions was added to the tubes and incubated at 65 °C for 20 minutes with occasional inverting the tubes. Immediately 600 µl of chloroform was added and gently emulsified by inversition (3-5 times). Then the tubes were centrifugeted at 10.000 rpm for 5 minutes. After centrifugation, upper aqueous phase containing DNA was transferred to a new sterile tubes and 800 µl of fresh previously prepared precipitation solution (including 720 µl of sterile distilled water and 80 µl of precipitation solution for each individual) was added. Fallowing, the gently mixing by several inversition at room temperature, tubes were centrifugeted at 10.000 rpm for 5 min. Supernatant was removed and DNA pellet remained at the bottom of the tubes was dissolved in 200 µl 1.2M NaCl solution by gentle vortexing. Then 600 µl of cold ethanol was added to the tubes to precipitate to DNA at -20 °C for 3 hour. After that ethanol was poured, the tubes were lied on tissue paper to dry to remove ethanol from the DNA pellet. Lastly, DNA was dissolved in 100 µl sterile distilled water by gently vortexing. DNA samples was quantified at 230, 260 and 280 nm for detection of absorptions of RNA, DNA and protein parts respectively, if available in solution and run on 1 % agarose gel electrophoretically to confirm the presence of DNA.

2.3.2 Microsatellite amplification by PCR

Nine microsatellite loci isolated other cyprinid species were examined by PCR optimization for *Pseudophoxinus* species. However, only 6 primers were successfully optimized in *Pseudophoxinus* samples. The specific name, primer sequence, fluorescent

dye and PCR conditions are given in Table 2.2. PCR optimization was conducted using a gradient thermal cycler (HVD Life Science Model: CG1-96) with the annealing step set at 45–65 °C to determine optimum annealing temperature for all primers. Additional gradients were also run using $MgCl_2$ concentrations between 1 to 2.5 mM with 0.25 mM intervals. Figure 2.3 show PCR optimization products in 4% agoroze gel for six microsatellite primers.

For MFW-1 the reaction mixtures had a total volume of 25 µl including 0,2 mM dNTPs, 10x PCR buffer containing KCl, 1,75 mM $MgCl_2$, 20 pico mole for each primers, 0,5 units of Taq DNA polymerase (Fermentas), 50 ng of DNA. Reactions were denatured at 94 °C for 2 minutes, then carried out 35 cycles at 94 °C (40 s), annealing temperature 47 °C (40 s), 72 °C (90 s), followed by a final extension of 72 °C 5 minutes.

For SarN7G5, SarN7F8, SarN2F11a, SarN2F11b and SarN7K4; the reaction mixtures had a total volume of 25 µl including 0,2 mM dNTPs, 10x PCR buffer containing (NH4)2SO4, 1,5 mM MgCl2, 12,5 pico mole forward and reverse primers, 0,5 units of Taq DNA polymerase (Fermentas), 50 ng of DNA. Reactions were denatured at 94 °C for 2 minutes, then carried out 35 cycles at 94 °C (40 s), annealing temperature (47 °C) (40 s), 72 °C (40 s), followed by a final extension of 72 °C (1 minutes). After the optimization of the primers all the *Pseudophoxinus* populations were examined for the 6 primers. In order to check whether PCR products were amplified, a 4% agorose gel was used for electrophoresis and photographed under uv light condition.

After the optimization populations were examined using previously labeled microsatellite primers with fluorescence dye. Three different fluorescence dyes were used for the primers (HEX, 6-FAM, TET). Microsatellite primers were labeled according to their expected allele size recorded in previous studies in the literature. Primers having near allele size were labeled with different fluorescence dye in order to prevent overlapping. Mixtures of six fluorescently labeled PCR products were run simultaneously on ABI 310 genetics analyzer for genotyping. Sizing was obtained by

comparison with the internal standard Tamra 500 (Applied Biosystem). Genotyping was performed by RefGen Genetic Research and Biotechnology Company (http://www.refgen.com/). Scoring was performed using the program Gene Scan 3.1 (Applied Biosystem).

Figure 2.4 show the electropherogram of GeneScan 3.1 fragment analysis results showing PCR amplification for six microsatellite primers labeled different fluorescence dyes. Upper window of the electropherograms show peaks of detected fragments and lower window is tabular data containing the estimated size (in base pairs), peak area and height of detected fragments. There are three fluorescence dyes highlighted as blue, green and yellow in tabular data corresponding to 6-FAM, TET and HEX respectively. Table 2.2 shows fluorescence dye of each primer. Labeling with different fluorescence dye prevent overlapping of the allele peaks in the electropherogram.

Detected alleles having more than 200 relative fluorescence units (RFU) were evaluated as correct fragment peak (Sprecher *et al*. 1996). Alleles differing in size by even more bases were assessed as heterozygote allele. The maximum size difference between heterozygote alleles was evaluated as 10 base pairs. Peaks out of the expected allele size more than 10 bases are evaluated as non specific bands (Figure 2.4). Adding an extra base to detected microsatellite primers by Tag DNA Polymerase (Slippage) may cause differing in size by one base. Such extra peak is named as shadow peak. To make correct selection and eliminate shadow peaks, height and frequency of peaks were used as selection criteria. Alleles having the highest peak were firstly chosen for each primer and individual of a population. Then alleles having the highest frequencies among the alleles in the population were designated as correct allele size for the populations. (Personal communication RefGen Staff; Ginot *et al*. 1996).

2.3.3 Statistical Analysis

Microsatellite loci were characterized as allele frequencies and the number of alleles per locus and population, were estimated for each locus, overall locus and population using FSTAT program 2.9.3.2 (Goude 2001). The observed and expected heterozygosity (gene diversity) under Hardy-Weinberg Equilibrium, deviations from Hardy Weinberg equilibrium, and inbreeding coefficient (fixation index - Fis), genetic differentiation test significant by pairwise Fst measurements per population and locus were calculated using GenAlEx version 6.1 software (Peakall and Smouse 2007). For each population, individual heterozygosity was calculated as the number of loci at which the individual was heterozygous, divided by the total number of loci at which an individual was scored.

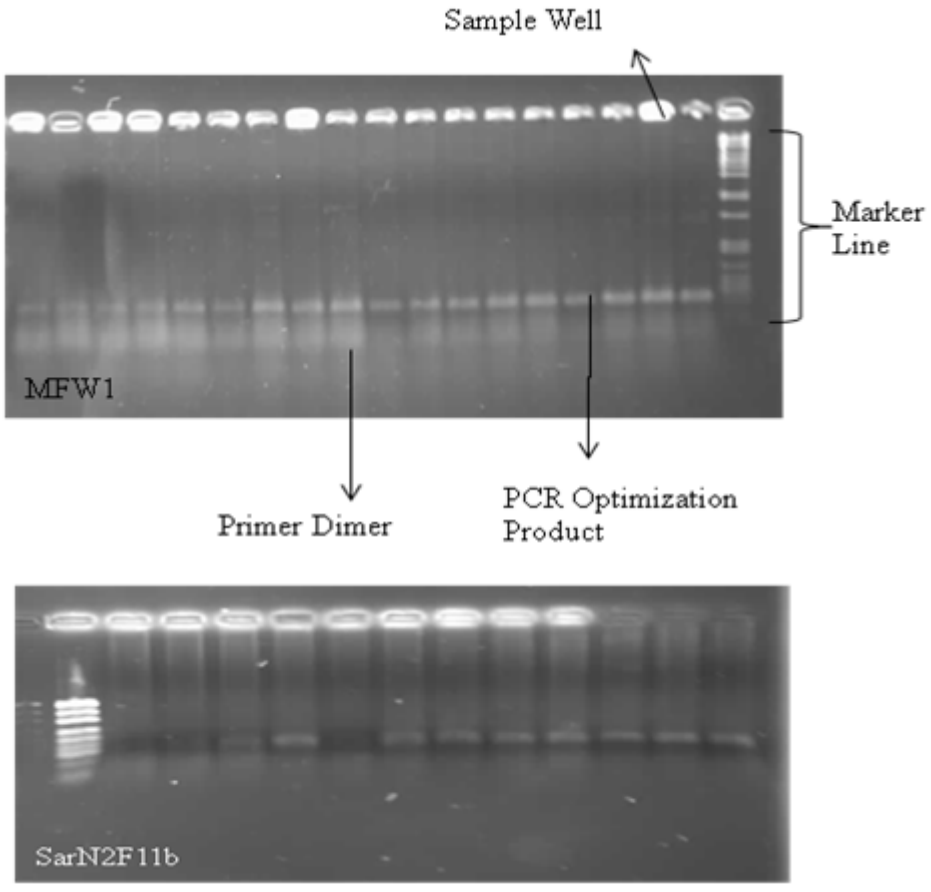

Figure 2.3: Bands that refer to six microsatellite primers optimized in gradient PCR.

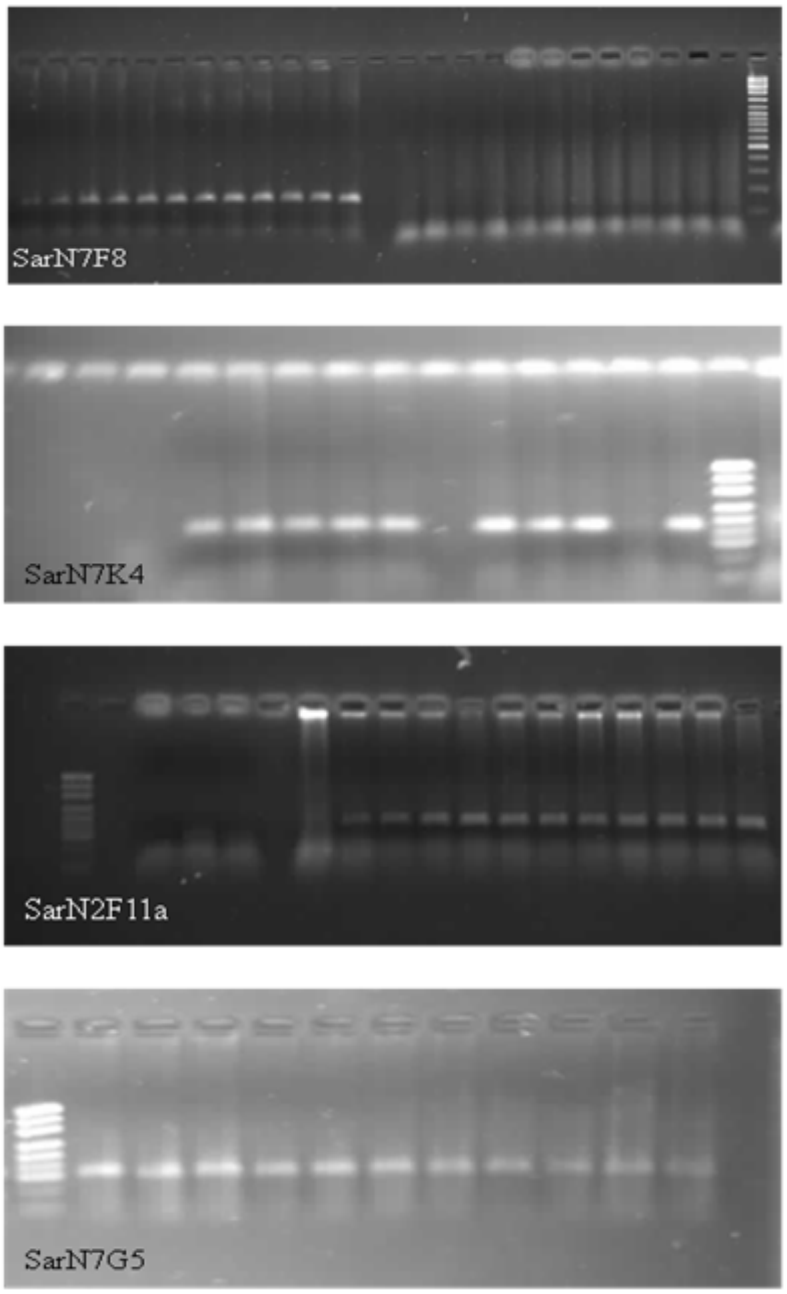

Figure 2.3: Continued.

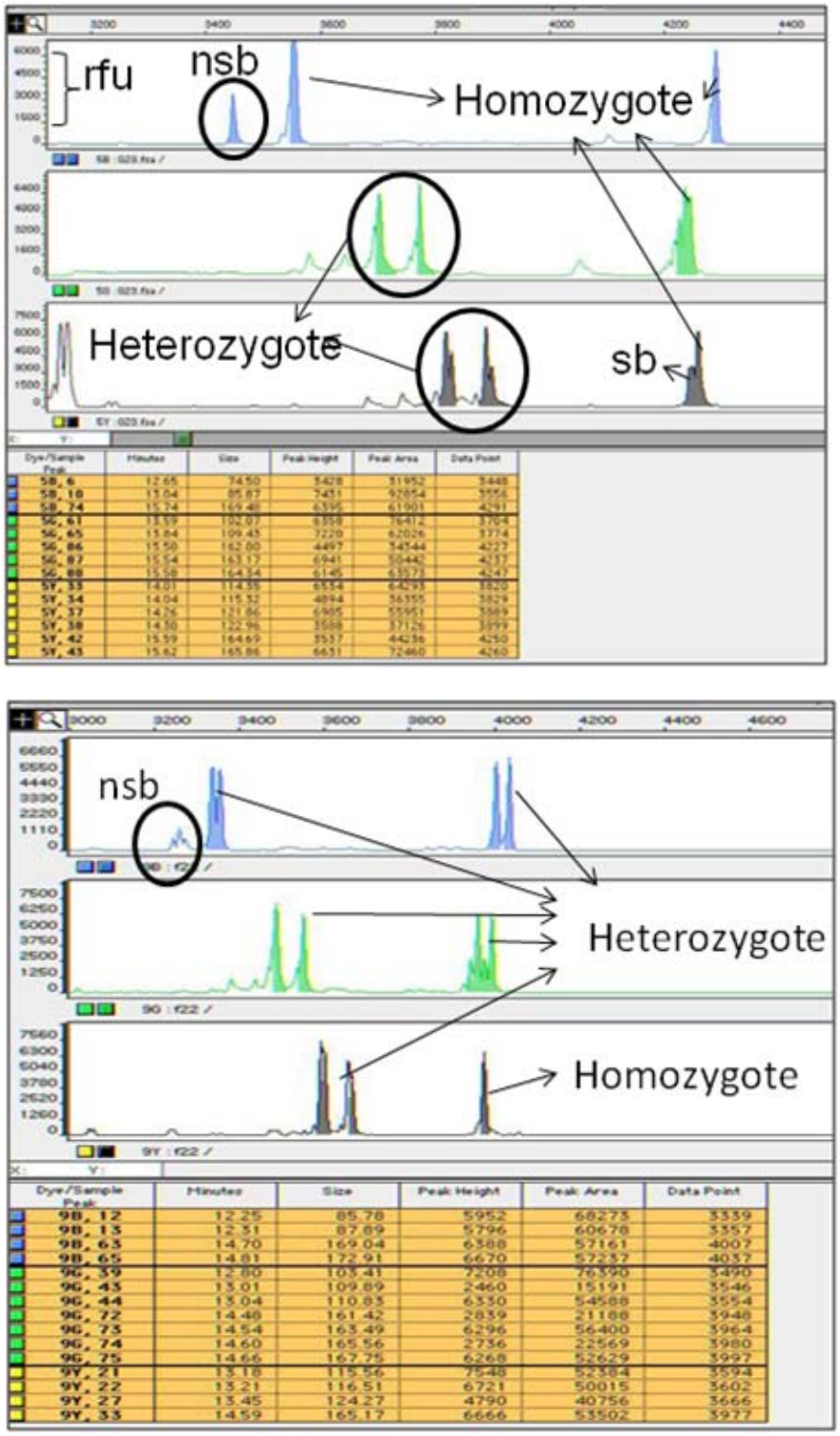

Figure 2.4: Two electropherogram of GeneScan fragment analysis results showing PCR amplification for six microsatellite primers labeled different fluorescence dyes (nsb is nonspecific band, sb is shadow band and rfu is relative fluorescence unit).

Table 2.2: The specific name, sequence, fluorescence dye label, forward and reverse primer sequences, repeat motifs, annealing temperature and $MgCl_2$ concentration (mM), PCR results, and reference of the primers used for the optimization.

Locus Name	Label	Forward and riverse primer	Ta °C/ MgCl2	Repeat Motif	PCR Results	Primer reference
MFW-1	HEX	F:GAGCTTCAGCACCGAGGAC R:GTCCAGACTGTCATCAGGAG	47; 1, 75	CA	+	Crooijmans et al. 1997
SarN7G5	6-FAM	F:GAGCTTCAGCACCGAGGAC R:CTACATGACAAGCATCTGCAGTAA	47; 1, 5	(TG)15CG(TG)2CGTGCG(TG)3	+	Mesquita et at. 2003
SarN7K4	TET	F:CATGTTTCCACATCTGAGCTAAAA R:ACGAGCATCAGTATCCAGAGACAC	47; 1, 5	(TG)16	+	Mesquita et at. 2003
SarN7F8	HEX	F: ACATTCCCTCTCTCACTTTCTGTC R: CTGGAGACCACAGTCAGGTAAATC	47; 1, 5	AG)4CAAGTGAGAGG(AG)3GGTG AG)3TGAGTG(AG)4AAAGCA(AG)2	+	Mesquita et at. 2003
SarN2F11a	6-FAM	F:GACCACGACACACTGAAA R: CCAGCGTTCCTCTACATCA	47; 1, 5	(CA)23N41(AC)10	+	Mesquita et at. 2003
SarN2F11b	TET	F: GAACAAACATCACTGAAGCACTCT R:ACGTCAGACTTCAGGCATCC	47; 1, 5	(AC)10	+	Mesquita et at. 2003
LceCb	Non Labeled	F:AGCCATTTGCCTTCATATTC R:CACAAACGCGGCATATC	-	(GT)10(GA)21	-	Larno et al. 2005
Lco4	Non Labeled	F:ATC AGG TCA GGG GTG TCA CG R:TGT TTA TTT GGG GTC TGT GT	-	[GT]5ATTTT [GT]5[GA]11	-	Turner et al. 2004
Gob12	Non Labeled	F:AAGGAAATGCAGAATCACAAAATTAC R:GAACTTGCAAAATAGCAGGGTG	-	(CA)7AA(CA)19	-	Knapen et al. 2006

2.4 Allozyme Analyses

For the allozyme analyses, around 50 µg muscle were homogenized in equal volume distilled water and centrifugeted at 5 000 rpm at 4 °C for 5 minutes. The supernatant was stored in capillary tubes at – 80 °C until analyses. Genotypes of individuals were scored by 9% starch gel electrophoresis. The gel consisted of 18 g starch 25 ml buffer and 375 ml of distilled water. The mixture was heated to boiling point in to a 2 L round bottom flask over Bunsen burner. Then air bubbles were removed from the solution by evacuating air from the solution with a water pump. After that the solution was poured into a glass plate having plastic bars and it was allowed to cool for about 20 min. When cool enough, upper part of the gel was removed to make surface of the gel smooth. Samples from the supernatants were soaked in to heavy filter papers in order to load in to the gel. Optimum electrophoresis conditions were 60 mamp, 200 V for 3 hours. Electrophoresis was carried out at 4 °C at constant temperature (Simith T.B. and Wayne 1996).

Allelic designation follows the use of Paustor (1990). Seven Enzyme systems corresponding to 10 loci were analyzed, electrophoretic buffer used are illustrated in Table 2.3. Allelic frequencies observed and expected heterozygosity, inbreeding coefficient (fixation indices - Fis), genetic identity and distance matrix and an UPGMA dendogram were computed for each populations using POPGENE32 software (Nei, 1973). For each population, individual heterozygosity was calculated as the number of loci at which the individual was heterozygous, divided by the total number of loci at which an individual was scored.

Table 2.3: Enzyme systems and corresponding electrophoretic buffers. Tissues: M = Muscle. Buffers: TC 7.5 = Tris-citrate pH 7.5 gel buffer and electrode buffer, TC 6.7 = Tris-citrate pH 6.7 gell buffer/ pH 6.4 electrode buffer (Pasteur et. al 1988).

Enzymes	IUBNC Number	Locus	Tissue	Buffer
Glucose-6-phosphate Isomerase	5.3.1.9	GPI-A	M	TC6.7
		GPI-B	M	TC6.7
Phosphoglucomutase	5.4.2.2	PGM	M	TC6.7
Malate Dehydrogenase	1.1.1.37	MDH-1	M	TC7.5
		MDH-2	M	TC7.5
L-Lactate Dehydrogenase	1.1.1.27	LDH-A	M	TC6.7
		LDH-B	M	TC6.7
Phosphogluconate Dehydrogenase	1.1.1.44	PGDH	M	TC6.7
Isocitrate Dehydrogenase	1.1.1.42	ICD	M	TC7.5
Glucose-6- phosphate Dehydrogenase	1.1.1.49	GPDH	M	TC6.7

CHAPTER 3

RESULTS

3.1 Geometric Morphometric Analyses.

The structure of among-population shape variation was assessed by canonical variate analyses (CVA) of partial warps and uniform components (Figure 3.1). The first two canonical variate axis of CVA explain 83.31% of the total variation in shape among eight populations. The first Canonical Variant (CV) axis describes 74.42% of that variation, and it clearly separates İnsuyu and Lake Gök populations of *P. crassus*. *P. battalgili* and *P. egridiri* populations seem to be overlapped with each other and they are separated from all of *P. crassus* and *P. sp* populations. The second CV axis that describes 8.89% of that variation clearly separates *P. sp* and *P. crassus* populations. The majority of *P. egridiri* population seems to be clustered separately from *P. battalgili* populations (Figure 3.1).

The ordination for the first and the second Principle Component (PC) axes (Figure 3.2) represents the partial warps and affine (uniform) shape variation. The first PC axis explains 40.09%, the second one comprises 21.02%, the third one is 9.44% and the fourth one is 6.70% of the total shape variance among eight populations. Thus, more than 77% of the variance is included within the first four principle component, which is sufficient for interpreting the shape variation within the studied data.

These partial warp and uniform components were then used for a multivariate analysis. Pair-wise test comparison performed by Morpheus (Slice 1998) for all the populations showed that Seydisehir River and Lake Sugla populations and also Lake Çavusşçu and Oymapınar Dam populations of *P. battalgili* were identical (Table 3.1).

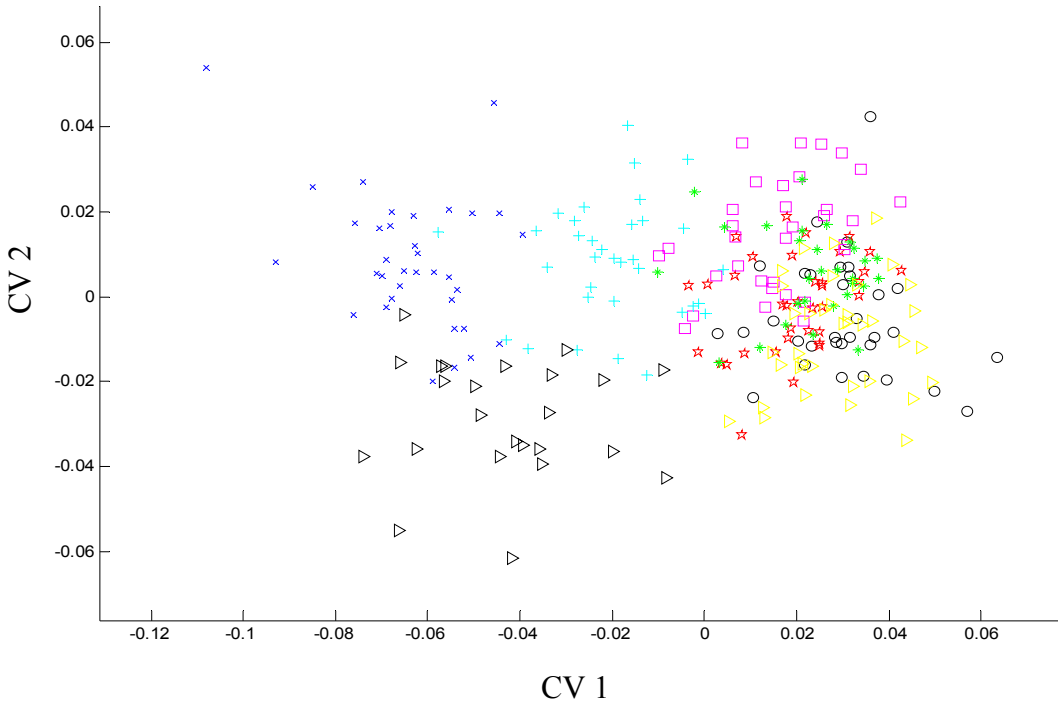

Figure 3.1: Scatter of 261 individuals on Canonical variate 1 and 2 axes based on the Procrustes distances from mean shape of the sampled *Pseudophoxinus* populations. (O: *P. battalgili*, Oymapınar Dam (n=33); ☆ *P. battalgili,* Lake Suğla (n=36); ✱ *P.battalgili,* Taşağıl River (n=29); Δ *P. battalgili,* Lake Çavuşçu (n=37); Δ *P. sp*, Eflatun River (n=24); **x** *P. crassus*, Lake Gök (n=35); + *P. crassus*, İnsuyu River (n= 34); □ *P. egridiri*, Lake Eğirdir (n=33).

Table 3.1: Pair-wise test comparison for all the population of *Pseudophoxinus*. (N = 261, α = 0,05, * P<0.05) Morpheus (Slice 1998).

	Oymapınar	Gök	Suğla	Eğridir	İnsuyu	Taşağıl	Çavuşçu	Eflatun
Oymapınar	--							
Gök	0,001*	--						
Suğla	0,001*	0,001*	--					
Eğridir	0,001*	0,001*	0,001*	--				
İnsuyu	0,001*	0,001*	0,001*	0,001*	--			
Taşağıl	0,001*	0,001*	0,322	0,001*	0,001*	--		
Çavuşçu	0,008	0,001*	0,001*	0,001*	0,001*	0,001*	--	
Eflatun	0,001*	0,001*	0,001*	0,001*	0,001*	0,001*	0,001*	--

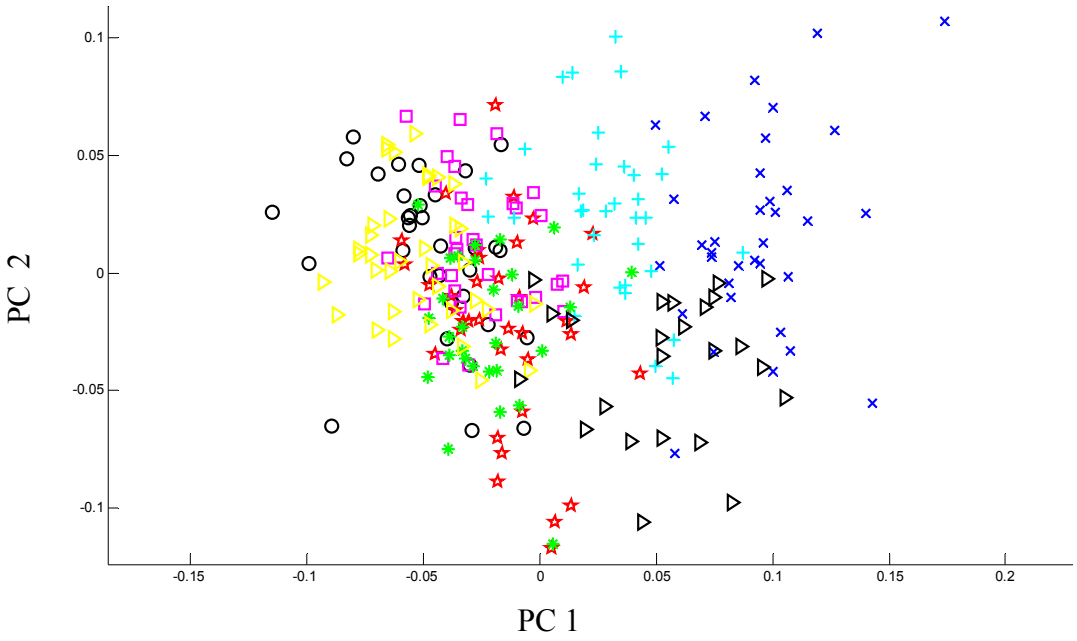

Figure 3.2: Scatter of 261 individuals on Principle Component variate 1 and 2 axes based on the Procrustes distances from mean shape of the sampled *Pseudophoxinus* populations. (O: *P. battalgili*, Oymapınar Dam (n=33); ☆ *P. battalgili,* Lake Suğla (n=36); ✱ *P. battalgili,* Taşağıl River (n=29); Δ *P. battalgili,* Lake Çavuşçu (n=37); Δ *P. sp*, Eflatun River (n=24); x *P. crassus*, Lake Gök (n=35); + *P. crassus*, İnsuyu River (n= 34); □ *P. egridiri*, Lake Eğridir (n=33).

Cartesian Transformation Grids (CTG) show overall shape differences of each population from the consensus shape are presented in Fig. 3.3. *P. battalgili* populations have elongated head formation than other groups with relatively wider eye circle and longer mouth formation. On the other hand opercular structure seems to be narrower than the other groups. *P. crassus* populations tend to be deeper head structure with the eye positioned relatively closer to the tip of the snout. They have narrower eye circle, shorter mouth formation and wider operculum structure. The relative position of upper pectoral fin is more forward in *P. crassus* than *P. battalgili*. *P. sp* have relatively wider and sharper mouth and head structure than *P. crassus*. *P. egridiri* seems to be consistent mouth and eye formation with consensus form, except for pectoral fin located in upper position. Picture of an individual from each population is given in figure 3.4.

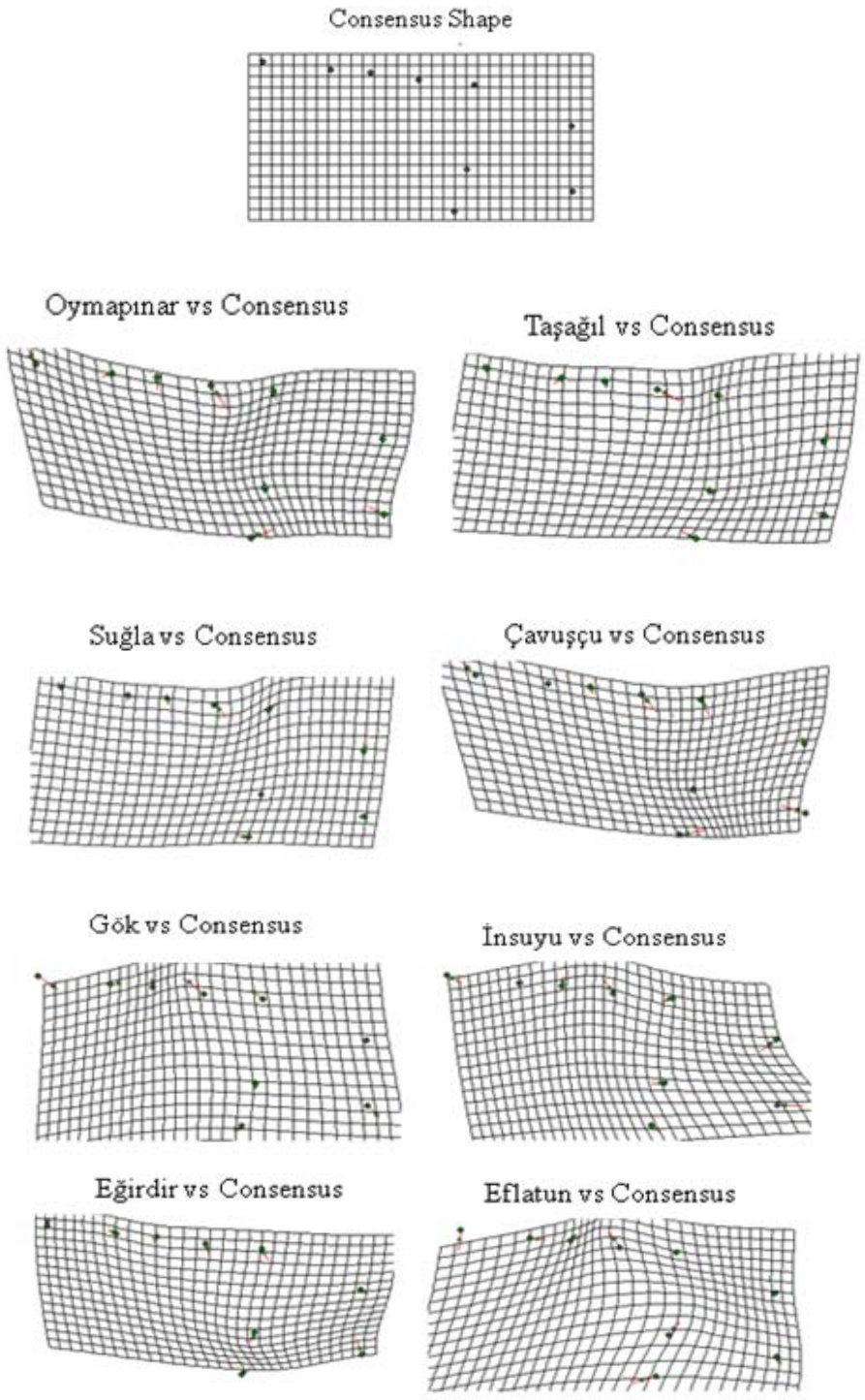

Figure 3.3: Cartesian transformation grids depicting the mean shape of all specimens examined, and the relative shape differences between the consensus and each of the *Pseudophoxinus* populations. Deformations are exaggerated 1.4 times for Lake Gök, 2 times for Eflatun River and 3 times rest of the populations.

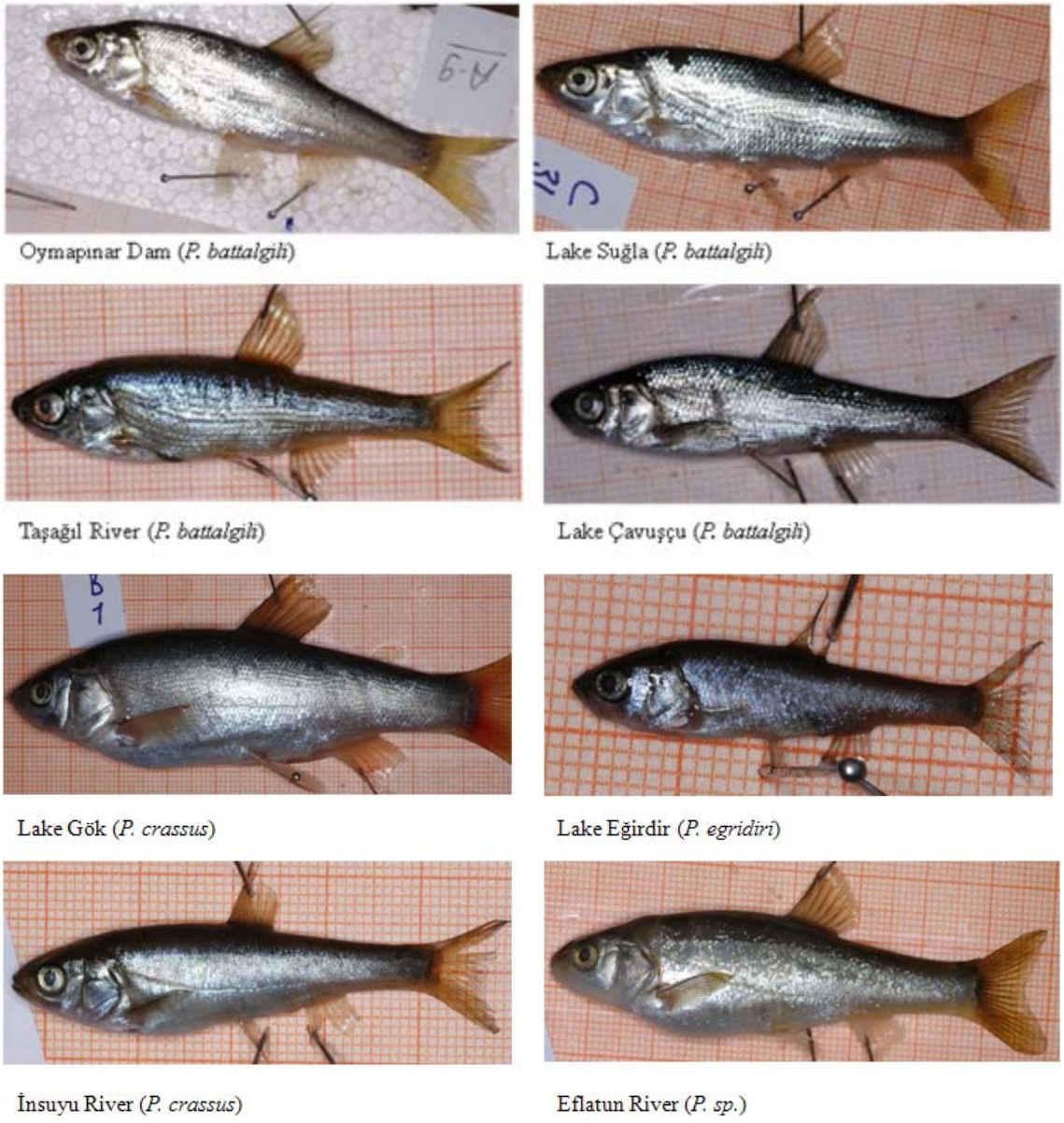

Figure 3.4: Picture of an individual from each population.

Generalized Goodall F-test were used to detect whether there is any significant variation in shape with size among the populations by regressing landmark coordinate data on centroid size (TPSRegr). Centroid size is the square root of the sum of the squared distances of the landmarks from the centroid (Bookstein 1991). Results showed that shape variate as a function of size when all the populations are combined (Generalized

Goodall F-test; F = 5.3439, df = 14.3626: P = 0.0000- statistical test were done using TpsReg software (Rohlf 2003).

UPGMA dendogram illustrated in Figure 3.5 showed that Suğla and Taşağıl population are closely associated with each other. *P. battalgili* and *P. crassus* populations show diverse clustering in the dendogram analyses. Population from İnsuyu (*P.crassus*) seems to be more related with *P. battalgli* populations than Lake Gök population. Lake Gök (*P. crassus*) and Eflatun River (*P. sp*) populations are clustered in the same groups.

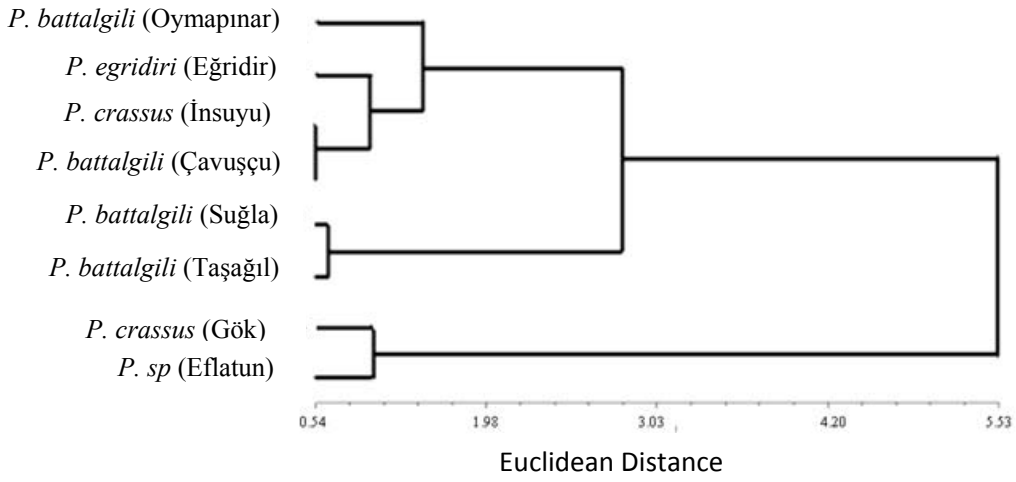

Figure 3.5: UPGMA dendrogram resulting from cluster analysis of Procrustes distances among mean shape of each *P. battalgili* populations.

3.1.1 Geometric Morphometric Analyses for *P. battalgili*

Canonical variate analyses (CVA) among *P. battalgili* populations are illustrated in Figure 3.6. The first two canonical variate axis explains 90.72 % of the total variation in shape among four populations. Lake Çavuşçu population is clustered separately in CVA 1 axis that describe 58.65 % of the total variation. The majority of Lake Suğla and Taşağıl River populations are overlapped. The second CV axes describes 32.07 % of that variation and it separates partially Lake Çavuşçu and Oymapınar Dam populations.

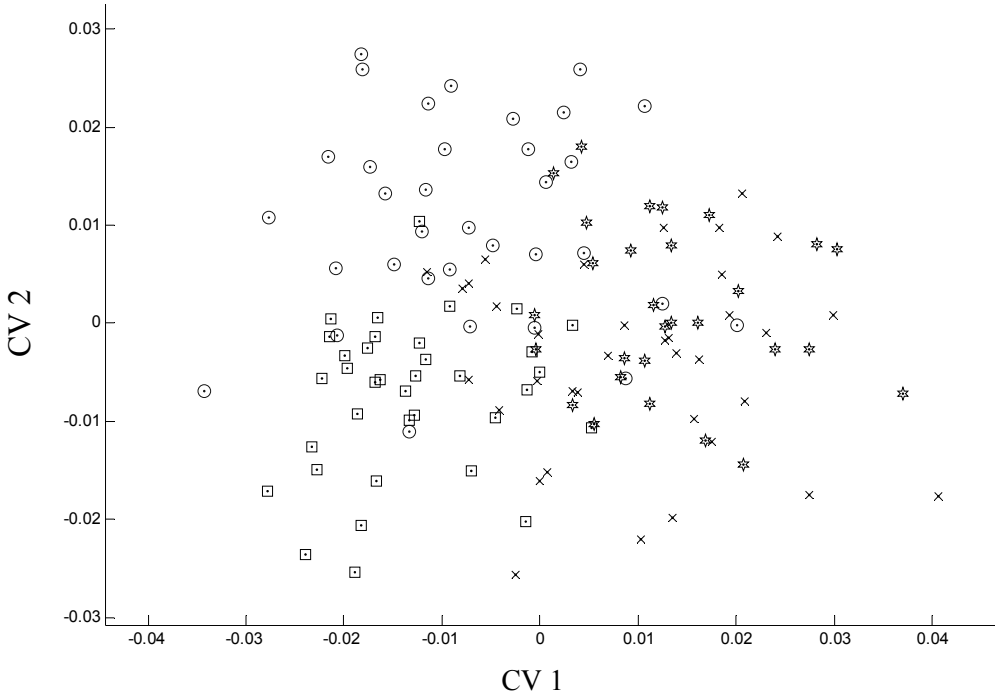

Figure 3.6: Scatter of individuals on Canonical variate 1 and 2 axes based on the Procrustes distances from mean shape of 4 *P. battalgili* populations. o Oymapınar Dam (n=33); X Lake Suğla (n=36); □ Lake Çavuşçu (n=37); ✯ Taşağıl River (n=29).

The ordination for the 1st and 2nd Principle Component analyses (PCA) is illustrated in Figure 3.7. The first PC axis constitutes 35.55% of the variance, the second PC axis comprises 14.81% of the variance, the third one is 10.71% and the fourth one is 10.14%. Thus, more than 71% of the variance is included within the first four principle component, which is sufficient for interpreting the shape variation.

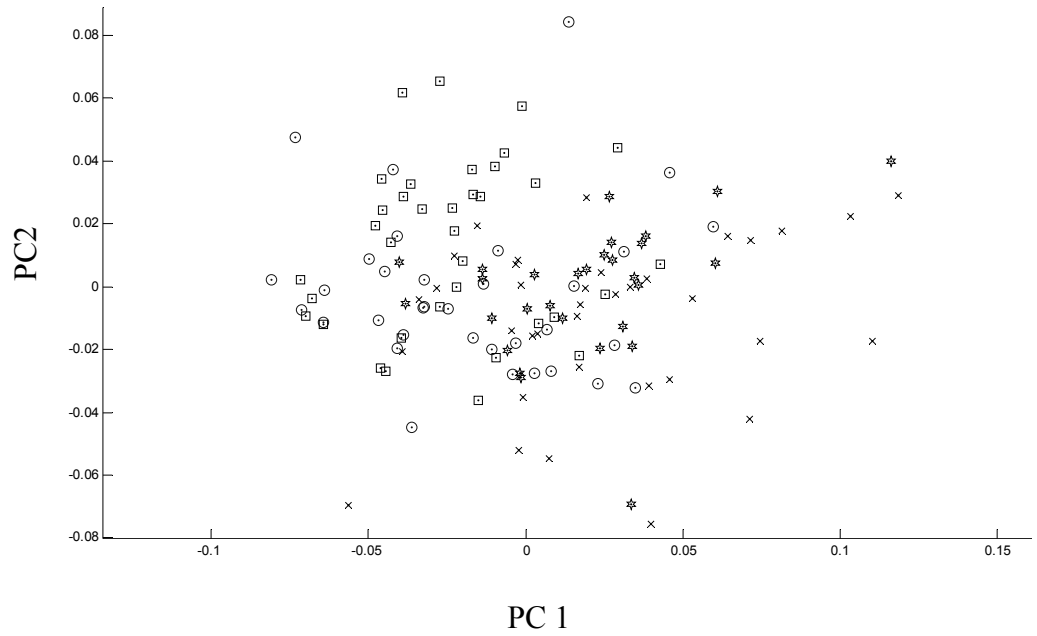

Figure 3.7: Scatter of individulas on Principle Component variates 1 and 2 axes based on the prucrusters distances from mean shape of 4 *P. battalgili* populations. **O** Oymapınar Dam (n=33); X Lake Suğla (n=36); □ Lake Çavuşçu (n=37); ✯ Taşağıl River (n=29).

Pair-wise test comparison between 4 population of *P. battalgili* show that Lake Suğla and Taşağıl River populations and Lake Çavuşçu and Oymapınar Dam populations are clustered in the same group (Table 3.2).

Cartesian Transformation Grids (CTG) depicting overall shape differences of each *P. battalgili* populations from the consensus shape are presented in Figure 3.8. Lake Çavuşçu population has relatively elongated head formation with wider operculum and longer mouth structure than other populations. Moreover, Lake Oymapınar and Lake Suğla populations have wider head structure than Taşağıl River population.

Table 3.2: Pair-wise test comparison between 4 population of *P. battalgili*. (N=135, α=0,05, * P<0.05) (Morpheus) (Slice 1998).

	Oymapınar Dam	Lake Suğla	Taşağıl River	Lake Çavuşçu
Oymapınar Dam	-	-	-	-
Lake Suğla	0,002*	-	-	-
Taşağıl River	0,002*	0,335	-	-
Lake Çavuşçu	0,011	0,001*	0,001*	-

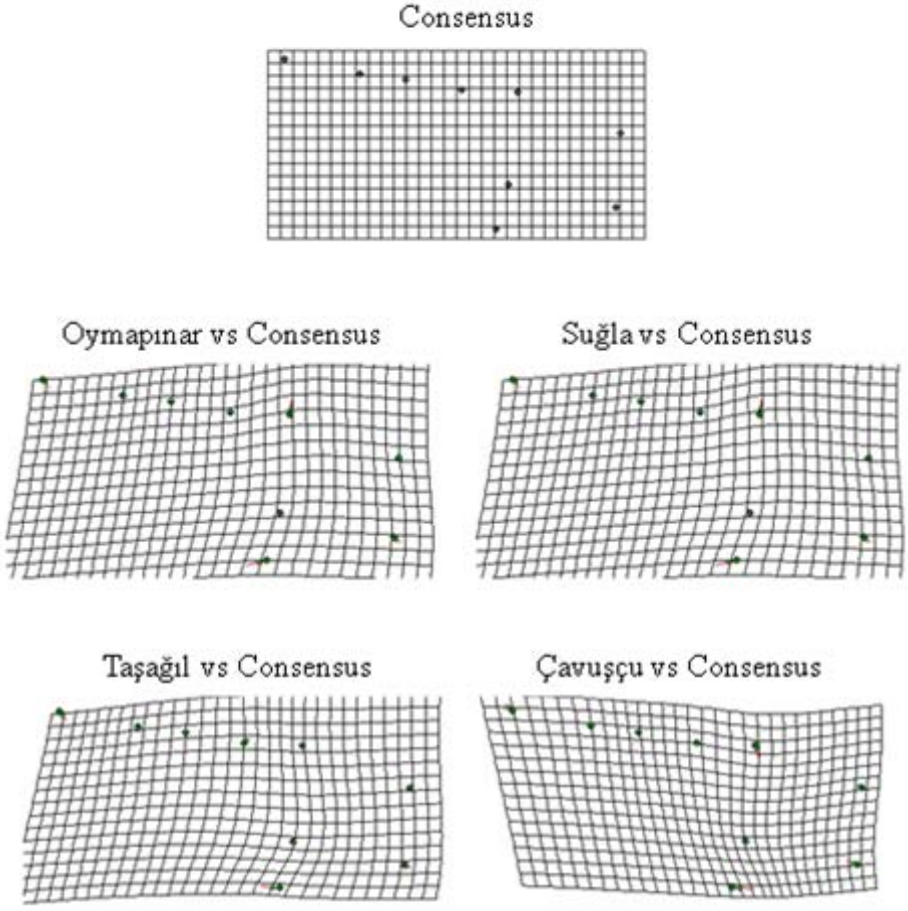

Figure 3.8: Cartesian transformation grids depicting the mean shape of all specimens examined, and the relative shape differences between the consensus and each of the 4 *P. battalgili* populations. Deformations are exaggerated 3 times.

UPGMA dendogram illustrated in Figure 3.9 showed that Suğla and Taşağıl population are closely associated with each other. Population from Oymapınar and Çavuşçu are clustered in separate groups.

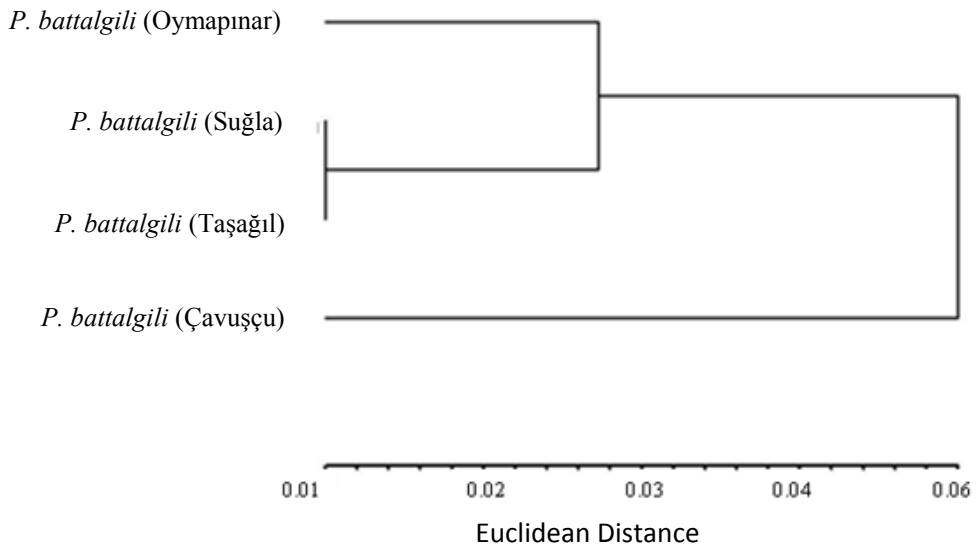

Figure 3.9: UPGMA dendrogram resulting from cluster analysis of Procrustes distances among mean shape of each *P. battalgili* populations.

Procrustes distances of each individual were calculated for *P. battalgili* populations in order to regress mean heterozygosity of individuals. Procrustes distance refers to the square root of the sum of squared differences between an individual and consensus configurations at centroid size. That quantity defines shape differences of an individual from a population to the consensus (mean) shape of the population. The consensus shape configurations were calculated for each population separately and deviation from the consensus form were listed as procrustes distances for each individual in Table 3.3. These values are considered as individual shape variation from the consensus shape and used for making correlation between shape variation and average heterozygosity of allozyme and microsatellite data for each individual.

Table 3.3: Procrustes distances (d^2) of each individual of *P. battalgili* populations.

	Oymapınar (n=33)	**Suğla (n=36)**	**Taşağıl (n=29)**	**Çavuşçu (n=37)**
1	0,0037	0,00545	0,0049	0,0052
2	0,0060	0,01193	0,0123	0,0055
3	0,0034	0,00258	0,0030	0,0020
4	0,0024	0,00448	0,0032	0,0017
5	0,0015	0,00977	0,0017	0,0023
6	0,0028	0,00284	0,0020	0,0049
7	0,0069	0,00167	0,0010	0,0033
8	0,0037	0,00429	0,0027	0,0046
9	0,0076	0,00257	0,0018	0,0006
10	0,0143	0,00372	0,0025	0,0055
11	0,0064	0,00113	0,0009	0,0016
12	0,0087	0,00296	0,0031	0,0014
13	0,0012	0,00387	0,0029	0,0023
14	0,0011	0,00569	0,0055	0,0043
15	0,0026	0,00282	0,0052	0,0029
16	0,0050	0,00243	0,0014	0,0014
17	0,0030	0,0013	0,0006	0,0035
18	0,0015	0,00441	0,0028	0,0022
19	0,0027	0,00727	0,0023	0,0035
20	0,0054	0,00272	0,0022	0,0044
21	0,0074	0,00184	0,0018	0,0037
22	0,0070	0,00826	0,0044	0,0020
23	0,0030	0,00196	0,0010	0,0063
24	0,0063	0,00156	0,0010	0,0013
25	0,0037	0,00302	0,0022	0,0027
26	0,0062	0,00525	0,0012	0,0056
27	0,0032	0,00793	0,0029	0,0006
28	0,0029	0,00839	0,0020	0,0018
29	0,0032	0,00128	0,0076	0,0020
30	0,0010	0,00539		0,0036
31	0,0023	0,0016		0,0039
32	0,0026	0,00647		0,0028
33	0,0027	0,00912		0,0048
34		0,0032		0,0065
35		0,00716		0,0040
36		0,0057		0,0037
37				0,0028

Generalized Goodall F-test showed that shape variate as a function of size when all the *P. battaligili* populations are combined. However, there is no significant variation in shape with size within populations for *P. battaligili* (Table 3.4).

Table 3.4: Variation in shape as a function of size (centroid) within and among populations for *P. battaligili*. (Generalized Goodall F-test, TpsReg).

Populations	F	df	P
Oymapınar Dam	0.3937	14, 434	0.9765
Lake Suğla	1.3764	14, 476	0.1602
Taşağıl River	1.2707	14, 378	0.2228
Lake Çavuşçu	1.4390	14, 490	0.1307
Among populations	4.0849	14, 1862	0.0000*

3.1.2 Geometric Morphometric Analyses for *P. crassus*

Canonical variate analyses (CVA) among *P. crassus* populations are illustrated in Figure 3.10. The first two canonical variate axis explains 99 % of the total variation in shape among two populations. Lake Gök and İnsuyu River populations are clustered separately in CVA 1 axis that describes 98 % of the total variation.

The ordination for the first and the second principle component analyses (PCA) for *P. crassus* populations is illustrated in Figure 3.11. The first Principle Component axis constitutes 36.33% of the variance, the second one comprises 26.20% of the variance, the third one is 9.83% and the fourth one is 6.95%. Thus, more than 79.31 % of the variance is included within the first four principle component, which is sufficient for interpreting the shape variation within the studied data.

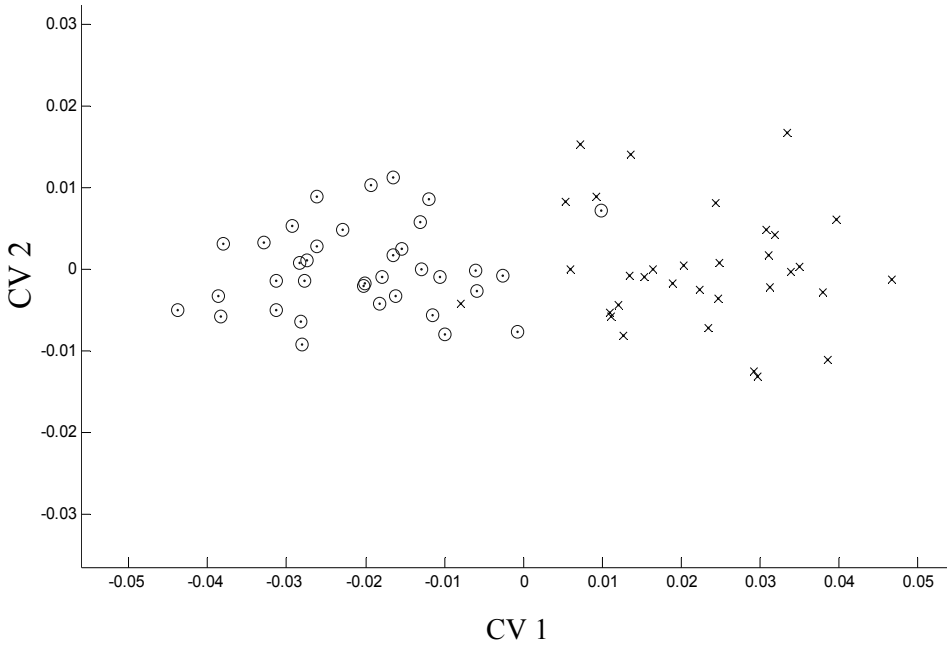

Figure 3.10: Scatter of individulas on Canonical variates 1 and 2 axes based on the prucrustes distances from mean shape of two *P. crassus* populations. O Lake Gök (n=35); X İnsuyu River (n=34).

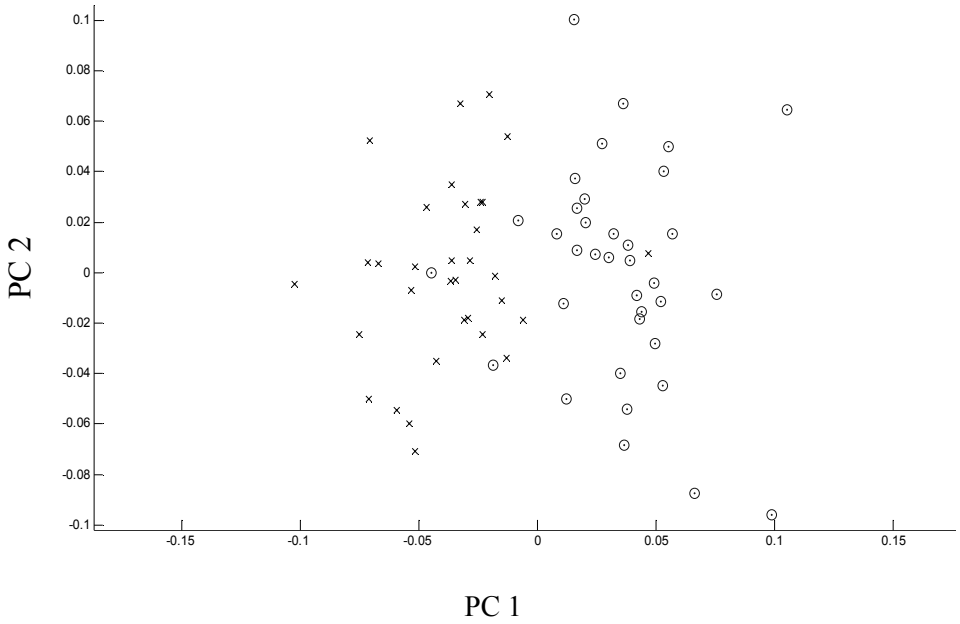

Figure 3.11: Scatter of individuals on Principle Component variate 1 and 2 axes based on the procrustes distances from mean shape of two *P. crassus* populations. **O** Lake Gök (n=35); X İnsuyu River (n=34).

Pair-wise test comparison between 2 populations of *P. crassus* show that Lake Gök and İnsuyu River populations are clustered in different groups (Table 3.5).

Table 3.5: Pair-wise test comparison between 2 populations of *P. crassus*. (N=69, α=0,05, *P< 0,05) (Morpheus) (Slice 1998).

	Lake Gök	İnsuyu River
Lake Gök	-	-
İnsuyu River	0,001*	-

Cartesian Transformation Grids (CTG) depicting overall shape differences of each *P. crassus* populations from the consensus shape are presented in Figure 3.12. While Lake Gök population shows wider and shorter structure in head formation, İnsuyu River populations have more elongated and narrower head formation.

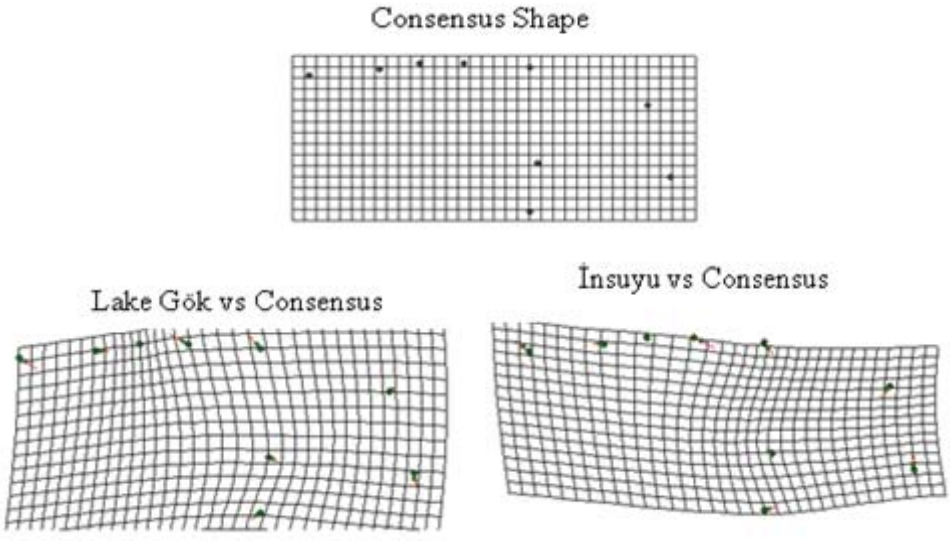

Figure 3.12: Cartesian transformation grids depicting the mean shape of all specimens examined, and the relative shape differences between the consensus and each of the two *P. crassus* populations. Deformations are exaggerated 3 times.

Procrustes distances (d^2) of *P. crassus* for both İnsuyu River and Lake Gök populations are given in Table 3.6. Generalized Goodall F-test showed that there is no significant variation in shape with size within and among populations for the *P. crassus* (Table 3.7)

Table 3.6: Procrustes distances (d^2) of each individual of *P. crassus* populations.

	Gök	**İnsuyu**	**Gök**	**İnsuyu**	
1	0,013151	0,00206	0,002534	0,007304	18
2	0,001747	0,00604	0,00264	0,002347	19
3	0,000978	0,00114	0,001734	0,003697	20
4	0,002841	0,00457	0,00541	0,003525	21
5	0,003973	0,00275	0,002768	0,001088	22
6	0,006585	0,00194	0,012128	0,001182	23
7	0,018741	0,0031	0,000918	0,002429	24
8	0,003495	0,00296	0,006548	0,001648	25
9	0,002616	0,00534	0,002392	0,005955	26
10	0,001839	0,00285	0,001846	0,00202	27
11	0,004131	0,00383	0,001604	0,002838	28
12	0,002546	0,00247	0,002326	0,005626	29
13	0,002681	0,00222	0,005917	0,008761	30
14	0,000885	0,00788	0,00269	0,003458	31
15	0,003077	0,00789	0,003747	0,001976	32
16	0,006411	0,00878	0,003775	0,002574	33
17	0,006219	0,00196	0,004744	0,011677	34
			0,009657		35

Table 3.7: Variation in shape as a function of size (centroid) within and among populations for *P. crassus* (Generalized Goodall F-test, TpsReg).

Populations	F	df	P
İnsuyu River	0.9613	14, 448	0.4924
Lake Gök	0.2140	14, 462	0.9990
Among populations	0.3393	14, 938	0.9888

3.1.3 Geometric Morphometric Analyses for *P. sp* and *P. egridiri*

Procrustes distances (d^2) of *P. sp* (Eflatun River) and *P. egridiri* (Lake Eğirdiri) populations are given in Table 3.8. Generalized Goodall F-test showed that there is no significant variation in shape with size within populations for both *P. sp* and *P. egridiri* (Table 3.9)

Table 3.8: Procrustes distances (d^2) of each individual of *P. sp* and *P. egridiri* populations.

	Eflatun	**Eğirdir**		**Eflatun**	**Eğirdir**
1	0,0035	0,0027	18	0,005215	0,0030
2	0,005884	0,0019	19	0,010186	0,0041
3	0,002749	0,0026	20	0,005886	0,0091
4	0,001951	0,0066	21	0,003528	0,0057
5	0,008339	0,0058	22	0,0034	0,0080
6	0,003679	0,0032	23	0,005655	0,0059
7	0,004937	0,0057	24	0,005613	0,0020
8	0,00556	0,0029	25		0,0019
9	0,002278	0,0030	26		0,0029
10	0,001934	0,0037	27		0,0028
11	0,006927	0,0023	28		0,0035
12	0,005921	0,0033	29		0,0031
13	0,003498	0,0023	30		0,0060
14	0,004946	0,0059	31		0,0006
15	0,005359	0,0038	32		0,0045
16	0,00985	0,0041	33		0,0037
17	0,006152	0,0021			

Table 3.9: Variation in shape as a function of size (centroid) within and among populations for *P. sp* and *P. egridiri* (Generalized Goodall F-test, TpsReg)

Populations	F	df	P
P. sp	0.9401	14, 308	0.5158
P. egridiri	1.3815	14, 434	0.1581

3.2 Microsatellite Analyses

Genetic variations among eight *Pseudophoxinus* populations were analyzed by determination of number of alleles, allele frequencies and expected and observed heterozygosity measures among populations.

3.2.1 Allele Frequency and Polymorphisms

Allele frequencies and the number of alleles per population for each *Pseudophoxinus* population given in Table 3. 10 and 3. 11 respectively were calculated using a computer program FSTAT (version 2.9.3) (Goudet, 1995).

The total number of allele detected from six microsatellite loci was 103. All the microsatellite loci were found to be polymorphic that varied between 2 and 12 allele per locus per population. Only SarN2F11a locus was monomorphic for *P. crassus* population from İnsuyu River. Moreover, *P. egridiri* population from Lake Egirdir and *P. crassus* population from İnsuyu River seems to be monomorphic for SarN2F11b locus with a common allele exceeding the frequency of 0.95 (Table 3.10). The mean number of alleles per locus ranged from 2.83 (*P. crassus* in İnsuyu River) to 7.17 (*P. sp* in Eflatun River). Alleles observed from SarN7K4 and MFW1 loci are more shared by the populations than other loci. SarN7K4 locus was not successfully amplified during the PRC process for *P. egridiri* population, so this locus was excluded from the all estimations.

Table 3.10: Estimated allele frequencies and total number of allele for each population at all microsatellite loci in 8 populations of *Pseudophoxinus* (* non amplified primer).

Locus/ Populations	**Estimated Allele Frequencies**								
	Allele	**Oymapınar**	**Gök**	**Suğla**	**Eğridir**	**Insuyu**	**Taşağıl**	**Çavuşçu**	**Eflatun**
SarN7F8	**158**								0.042
	160								0.042
	162		0.40			0.121			0.208
	164		0.60			0.879			0.625
	165						0.019		
	167	0.061							
	168								0.083
	169	0.606		0.569			0.685	0.865	
	170			0.111					
	171	0.242		0.306			0.241	0.135	
	173	0.091					0.037		
	175						0.019		
	179			0.014					
	186				0.081				
	188				0.823				
	190				0.097				
# of allele	**18**	**4**	**2**	**4**	**3**	**2**	**5**	**2**	**5**

Table 3.10: continued

Locus/ Populations	Estimated Allele Frequencies								
	Allele	**Oymapınar**	**Gök**	**Suğla**	**Eğridir**	**Insuyu**	**Taşağıl**	**Çavuşçu**	**Eflatun**
SarN7G5	**72**	0.015						0.041	
	74							0.810	
	78							0.054	
	80	0.030						0.027	
	82	0.045						0.054	
	84	0.121		0.042			0.222	0.162	
	86	0.667		0.861			0.704	0.473	
	87								0.021
	88	0.121		0.097			0.056	0.081	
	89								0.063
	90						0.019	0.027	
	91								0.042
	93				0.016				0.042
	94								0.021
	95				0.097				0.229
	97				0.548				0.250
	99				0.339				0.104
	101								0.063
	103								0.063
	105								0.063
	107								0.042
	111		0.029						
	113		0.043			0.030			
	115		0.029			0.015			
	117		0.486			0.455			
	119		0.257			0.045			
	121		0.157			0.242			
	123					0.212			
# of allele	**29**	**6**	**6**	**3**	**4**	**6**	**4**	**9**	**12**

Table 3.10: continued

Locus/ Populations	Estimated Allele Frequencies								
	Allele	**Oymapınar**	**Gök**	**Suğla**	**Eğridir**	**Insuyu**	**Taşağıl**	**Çavuşçu**	**Eflatun**
SarN7K4	**157**	0.015		0.014	*		0.037		
	159	0.045		0.083	*		0.056	0.014	0.022
	161	0.197		0.236	*		0.241	0.095	0.022
	163	0.167		0.181	*	0.136	0.130	0.176	0.043
	165	0.121		0.111	*	0.833	0.204	0.068	0.174
	167	0.091	0.267	0.111	*	0.030	0.148	0.122	0.196
	168		0.117		*				
	169	0.197	0.433	0.167	*		0.148	0.203	0.348
	170		0.183	0.028	*				
	171	0.152		0.028	*		0.037	0.297	0.087
	173			0.000	*			0.027	0.087
	175	0.015		0.042	*			0.022	0.022
# of allele	**13**	**9**	**4**	**11**	*	**3**	**8**	**9**	**9**

Locus/ Populations	Estimated Allele Frequencies								
	Allele	**Oymapınar**	**Gök**	**Suğla**	**Eğridir**	**Insuyu**	**Taşağıl**	**Çavuşçu**	**Eflatun**
SarN2F11b	**97**	0.032						0.027	
	99							0.027	
	101	0.532		0.514			0.537	0.473	
	103	0.113	0.857	0.097		**0.970**	0.074	0.054	
	104		0.114		0.016				0.083
	105	0.065	0.029	0.028		0.030	0.074	0.108	
	106				**0.953**				
	107	0.065		0.028					0.083
	108				0.031				
	109	0.097		0.250			0.278	0.230	0.542
	111	0.065		0.069			0.037	0.068	
	113							0.014	
	115	0.032							
	117								0.042
	119			0.014					0.125
	121								0.083
	127								0.042
# of allele	**18**	**8**	**3**	**7**	**3**	**2**	**5**	**8**	**7**

Table 3.10: continued

Locus/ Populations		Estimated Allele Frequencies							
	Allele	**Oymapınar**	**Gök**	**Suğla**	**Eğridir**	**Insuyu**	**Taşağıl**	**Çavuşçu**	**Eflatun**
SarN2F11a	**160**			0.028	0.047				
	161				0.094				
	162				0.734				0.024
	163				0.031				
	164	0.214			0.094			0.081	0.167
	165	0.036							
	166	0.643		0.931			0.944	0.838	0.690
	168	0.054		0.042			0.037	0.081	0.071
	170								0.048
	172	0.054					0.019		
	175		0.057						
	176		0.914			**1.00**			
	179		0.029						
# of allele	**13**	**5**	**3**	**3**	**5**	**1**	**4**	**3**	**5**

Locus/ Populations		Estimated Allele Frequencies							
	Allele	**Oymapınar**	**Gök**	**Suğla**	**Eğridir**	**Insuyu**	**Taşağıl**	**Çavuşçu**	**Eflatun**
MFW1	**112**	0.200			0.016		0.093	0.068	0.083
	114	0.317	0.029	0.473	0.063	0.015	0.204	0.527	0.194
	116	0.133	0.929	0.171	0.906	0.894	0.352	0.068	0.500
	118	0.117	0.043		0.016	0.091	0.019	0.054	0.139
	120	0.017		0.029			0.019	0.014	
	122	0.083		0.200			0.222	0.203	0.083
	123	0.017							
	124	0.067		0.100			0.093	0.054	
	126	0.017		0.014				0.014	
	128	0.033							
	144			0.014					
# of allele	**12**	**10**	**3**	**7**	**4**	**3**	**7**	**8**	**5**

Table 3.11: Number of alleles per population and locus

Locus/ Pop.	Oymapınar	Gök	Suğla	Eğridir	Insuyu	Taşağıl	Çavuşçu	Eflatun	Tot
Sar N7F8	4	2	4	3	2	5	2	5	**27**
Sar N7G5	6	6	3	4	6	4	9	12	**50**
Sar N7K4	9	4	10	*	3	8	8	9	**51**
SarN2F11b	8	3	7	3	2	5	8	7	**43**
SarN2F11a	5	3	3	5	1	3	3	5	**28**
MFW1	10	3	7	4	3	7	8	5	**47**
Total	**42**	**21**	**34**	**19**	**17**	**32**	**38**	**43**	**246**
Mean	**7**	**3.5**	**5.6**	**3.17**	**2.83**	**5.33**	**6.33**	**7.17**	

3.2.2 Allelic Heterozygosity

The observed and expected heterozygosity (gene diversity) under Hardy-Weinberg Equilibrium and fixation index calculated per population and locus and also their mean values with standard errors are given in Table 3.12. Calculations were performed using GenAlEx version 6.1 software (Peakall and Smouse 2007). Average expected heterozygosity (gene diversity) varied from 0.244 ± 0.103 (*P. crassus* from İnsuyu River) to 0.673 ± 0.056 (*P. sp* from Eflatun River). Mean gene diversity for all 8 populations was found to be 0,490 ± 0,038. Observed heterozygosity ranged from 0.160 (both *P. crassus* from İnsuyu River and *P. egridiri* with ± 0.104 and ± 0.095 standard error respectively) to 0.463 ± 0.127 (*P. battalgili* from Taşağıl). Fixation indices (F_{is}) calculated using only polymorphic loci for all the populations is given in Table 3.12. Positive values indicate a deficit and negative values indicate an excess of heterozygosity. Suğla population of *P. battalgili* has the lowest F_{is} value (0.154 ± 0.071) and Lake Gök and İnsuyu River populations of *P. crassus* have the highest F_{is} values (0.670±0.178 and 0.565±0.220 respectively) among the populations. The mean F_{is} values are significantly different than zero ($P < 0.01$) indicating heterozygote deficiency for all the populations. *P. crassus* from İnsuyu River is found the population having the lowest heterozygosity and gene diversity and one of the highest F_{is} values.

Table 3.12: Number of individuals analyzed (N), Number of Different allele (Na), Number of effective allele (Ne), Observed (Ho) and expected (He) heterozygosity, Unbiased Expected Heterozygosity (UHe =(2N / (2N-1)) * He), Shannon's Information Index (I), Fixation Index (Fis = (He - Ho) / He = 1 - (Ho / He)).

Oymapınar *P. battalgili*	**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**UHe**	**Fis**
Sar N7F8	33	4,000	2,283	1,035	0,303	0,562	0,571	0,461
Sar N7G5	33	6,000	2,096	1,092	0,030	0,523	0,531	0,942
Sar N7K4	33	9,000	6,501	1,966	0,818	0,846	0,859	0,033
SarN2F11b	31	8,000	3,125	1,560	0,258	0,680	0,691	0,621
SarN2F11a	28	5,000	2,145	1,047	0,214	0,534	0,544	0,599
MFW1	30	10,000	5,405	1,911	0,500	0,815	0,829	0,387
Mean	31,333	7,000	3,593	1,435	0,354	0,660	0,671	0,507
SE	0,843	0,966	0,775	0,178	0,111	0,059	0,060	0,123

Suğla *P. battalgili*	**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**UHe**	**Fis**
Sar N7F8	36	4,000	2,325	0,986	0,417	0,570	0,578	0,269
Sar N7G5	36	3,000	1,329	0,488	0,278	0,247	0,251	-0,123
Sar N7K4	36	10,000	6,612	2,035	0,694	0,849	0,861	0,182
SarN2F11b	36	7,000	2,919	1,359	0,528	0,657	0,667	0,197
SarN2F11a	36	3,000	1,151	0,299	0,083	0,132	0,133	0,367
MFW1	35	7,000	3,302	1,432	0,457	0,697	0,707	0,344
Mean	35,833	5,667	2,940	1,100	0,410	0,525	0,533	0,206
SE	0,167	1,145	0,812	0,263	0,086	0,113	0,115	0,073

Table 3.12: continued

Taşağıl *P. battalgili*	**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**UHe**	**Fis**
Sar N7F8	27	5,000	1,889	0,872	0,519	0,471	0,479	-0,102
Sar N7G5	27	4,000	1,825	0,816	0,111	0,452	0,461	0,754
Sar N7K4	27	8,000	5,740	1,861	0,815	0,826	0,841	0,013
SarN2F11b	27	5,000	2,646	1,197	0,519	0,622	0,634	0,166
SarN2F11a	27	3,000	1,119	0,250	0,111	0,106	0,108	-0,045
MFW1	27	7,000	4,301	1,614	0,481	0,767	0,782	0,373
Mean	27,00	5,50	3,034	1,128	0,463	0,549	0,559	0,158
SE	0,000	0,67	0,810	0,236	0,127	0,103	0,105	0,139

Çavuşçu *P. battalgili*	**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**UHe**	**Fis**
Sar N7F8	37	2,000	1,305	0,396	0,000	0,234	0,237	1,000
Sar N7G5	37	9,000	3,675	1,697	0,351	0,728	0,738	0,517
Sar N7K4	37	8,000	5,276	1,807	0,730	0,810	0,822	0,100
SarN2F11b	37	8,000	3,364	1,526	0,459	0,703	0,712	0,346
SarN2F11a	37	3,000	1,398	0,556	0,000	0,285	0,289	1,000
MFW1	37	8,000	2,992	1,457	0,459	0,666	0,675	0,310
Mean	37,000	6,333	3,002	1,240	0,333	0,571	0,579	0,545
SE	0,000	1,229	0,611	0,248	0,117	0,101	0,102	0,154

Lake Gök *P. crassus*	**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**UHe**	**Fis**
Sar N7F8	35	2,000	1,923	0,673	0,000	0,480	0,487	1,000
Sar N7G5	35	6,000	3,028	1,329	0,571	0,670	0,680	0,147
Sar N7K4	30	4,000	3,267	1,277	0,633	0,694	0,706	0,087
SarN2F11b	35	3,000	1,336	0,482	0,000	0,251	0,255	1,000
SarN2F11a	35	3,000	1,190	0,347	0,000	0,160	0,162	1,000
MFW1	35	3,000	1,156	0,305	0,029	0,135	0,137	0,789
Mean	34,167	3,500	1,983	0,735	0,206	0,398	0,404	0,670
SE	0,833	0,563	0,386	0,187	0,126	0,103	0,104	0,178

Table 3.12: continued

İnsuyu River *P. crassus*	**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**UHe**	**Fis**
Sar N7F8	33	2,000	1,271	0,369	0,000	0,213	0,216	1,000
Sar N7G5	33	6,000	3,189	1,341	0,515	0,686	0,697	0,249
Sar N7K4	33	3,000	1,401	0,530	0,333	0,286	0,290	-0,165
SarN2F11b	33	2,000	1,062	0,136	0,000	0,059	0,060	1,000
SarN2F11a	33	1,000	1,000	0,000	0,000	0,000	0,000	#N/A
MFW1	33	3,000	1,238	0,382	0,030	0,192	0,195	0,842
Mean	33,000	2,833	1,527	0,460	0,146	0,239	0,243	0,585
SE	0,000	0,703	0,338	0,192	0,091	0,099	0,101	0,213

Lake Eğirdir *P.egridiri*	**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**UHe**	**Fis**
Sar N7F8	31	3,000	1,444	0,590	0,194	0,307	0,313	0,371
Sar N7G5	31	4,000	2,353	0,989	0,613	0,575	0,584	-0,066
Sar N7K4	0	0,000	0,000	0,000	0,000	0,000	0,000	
SarN2F11b	32	3,000	1,099	0,219	0,031	0,090	0,092	0,654
SarN2F11a	32	5,000	1,786	0,922	0,031	0,440	0,447	0,929
MFW1	32	4,000	1,211	0,392	0,094	0,174	0,177	0,462
Mean	26,333	3,167	1,315	0,519	0,160	0,265	0,269	0,470
SE	5,270	0,703	0,322	0,160	0,095	0,089	0,090	0,150

Eflatun *P .sp*	**N**	**Na**	**Ne**	**I**	**Ho**	**He**	**UHe**	**Fis**
Sar N7F8	24	5,000	2,250	1,092	0,000	0,556	0,567	1,000
Sar N7G5	24	12,000	6,776	2,172	0,875	0,852	0,871	-0,026
Sar N7K4	23	9,000	4,809	1,801	0,913	0,792	0,810	-0,153
SarN2F11b	12	7,000	3,000	1,478	0,250	0,667	0,696	0,625
SarN2F11a	21	5,000	1,951	0,977	0,143	0,488	0,499	0,707
MFW1	18	5,000	3,115	1,353	0,278	0,679	0,698	0,591
Mean	20,333	7,167	3,650	1,479	0,410	0,672	0,690	0,457
SE	1,909	1,167	0,745	0,183	0,158	0,056	0,057	0,183

3.2.3 Genetic Structures of *Pseudophoxinus*

Genetic structure of *Pseudophoxinus* populations was analyzed by Hardy-Weinberg tests and population differentiation measures of F_{st} statistics. Measurements were performed using GenAlEx version 6.1 software (Peakall and Smouse 2007).

Deviations from Hardy Weinberg calculated for polymorphic locus at 0,01 and 0,001 level per locus and population are given in Table 3. 13. Populations show variable deviation from Hardy Weinberg equilibrium in locus level. *P. battalgili* from Lake Çavuşçu and *P. crassus* from Lake Gök show significant deviation from Hardy-Weinberg Equilibrium that favor homozygosity for all loci. On the other hand, Hardy-Weinberg deviation was in favor of heterozygosity except for at SarN7G5 and MFW1 locus for *P. battalgili* from Taşağıl River. Deviations favor homozygosity for all loci (except for SarN7K4) in *P. battalgili* from Oymapınar and *P. crassus* from İnsuyu populations.

Table 3.13: Significant from Hardy-Weinberg tests per locus and populations (m = monomorphic locus) (*P<0,01, **P<0,001).

	Oymapınar Dam (*P. battalgili*)			Lake Suğla (*P. battalgili*)		
	df	ChiSq	Prob	df	ChiSq	Prob
Sar N7F8	6	66,516	0,000**	6	36,639	0,000**
Sar N7G5	15	135,667	0,000**	3	0,937	0,817
Sar N7K4	36	29,546	0,768	45	83,081	0,000**
SarN2F11b	28	113,829	0,000**	21	39,064	0,010*
SarN2F11a	10	57,037	0,000**	3	36,072	0,000**
MFW1	45	86,173	0,000**	21	59,771	0,000**

Table 3.13: Continued

Taşağıl River (*P. battalgili*)				Lake Çavuşçu (*P. battalgili*)		
	df	ChiSq	Prob	df	ChiSq	Prob
Sar N7F8	10	4,758	0,907	1	37,000	0,000**
Sar N7G5	6	44,127	0,000**	36	199,410	0,000**
Sar N7K4	28	19,499	0,882	28	68,067	0,000**
SarN2F11b	10	14,958	0,134	28	121,837	0,000**
SarN2F11a	3	0,093	0,993	3	74,000	0,000**
MFW1	21	44,144	0,002*	28	110,279	0,000**

Lake Gök (*P.crassus*)				İnsuyu River (*P. crassus*)		
	df	ChiSq	Prob	df	ChiSq	Prob
Sar N7F8	1	35,000	0,000**	1	33,000	0,000**
Sar N7G5	15	48,846	0,000**	15	77,610	0,000**
Sar N7K4	6	44,038	0,000**	3	1,320	0,724
SarN2F11b	3	70,000	0,000**	1	33,000	0,000**
SarN2F11a	3	70,000	0,000**	m	--	--
MFW1	3	49,846	0,000**	3	33,009	0,000**

Lake Eğirdir (P.*egridiri*)				Eflatun River (*P.sp*)		
	df	ChiSq	Prob	df	ChiSq	Prob
Sar N7F8	3	14,157	0,003*	3	14,157	0,003*
Sar N7G5	6	6,762	0,343	6	6,762	0,343
Sar N7K4	m			m		
SarN2F11b	3	32,009	0,000**	3	32,009	0,000**
SarN2F11a	10	109,329	0,000**	10	109,329	0,000**
MFW1	6	23,210	0,001*	6	23,210	0,001*

Genetic differentiation was measured by computing F_{st} values between populations. F_{st} coefficients were given in Table 3.14. *P. egridiri*, *P. crassus* populations (İnsuyu and Lake Gök) and *P. sp* (Eflatun) were differentiated out from *P. battalgili* populations and each other. *P. battalgili* populations do not show genetic differentiation from one another. On the other hand *P. crassus* populations were not identical groups. There is a genetically divergence between İnsuyu River and Lake Gök populations.

Gene flow values (Nm) between populations are given in Table 3.15. High gene flow values were observed between *P. battalgili* populations. Suğla and Taşağıl populations have the highest value among *P. battalgili* populations. On the other hand there is comparatively lower gene flow detected between İnsuyu and Lake Gök population.

Table 3.14: Pairwise Fst values between populations.

	Oymapınar	Gök	Suğla	Egiridir	İnsuyu	Taşağıl	Çavuşçu	Eflatun
Oymapınar	-----							
Gök	0,289	-----						
Suğla	**0,030**	0,361	-----					
Egiridir	0,382	0,424	0,447	-----				
İnsuyu	0,359	0,092	0,439	0,525	-----			
Taşağıl	**0,029**	0,340	**0,017**	0,425	0,414	-----		
Çavuşçu	**0,027**	0,348	**0,037**	0,437	0,433	**0,031**	-----	
Eflatun	0,120	0,199	0,147	0,325	0,264	0,134	0,139	-----

Table 3.15: Pairwise Nm (gene flow) values between populations.

	Oymapınar	Gök	Suğla	Egiridir	İnsuyu	Taşağıl	Çavuşçu	Eflatun
Oymapınar	-----							
Gök	0,616	-----						
Suğla	8,141	0,458	-----					
Egiridir	0,406	0,340	0,321	-----				
İnsuyu	0,451	2,456	0,335	0,227	-----			
Taşağıl	9,056	0,495	13,94	0,345	0,363	-----		
Çavuşçu	8,750	0,475	5,793	0,327	0,333	7,533	-----	
Eflatun	1,845	1,005	1,523	0,520	0,704	1,646	1,578	-----

Genetic distinctness of *Pseudophoxinus* populations was also analyzed by contracting phylogenetic tree based on genetic distance among populations using Popgene32 software. The tree is illustrated in Figure 3.13. There are 2 distinctive groups among the populations. *P. battalgili* and *P. sp* populations are clustered in the same groups and *P. crassus* and *P.egridiri* are clustered in the other.

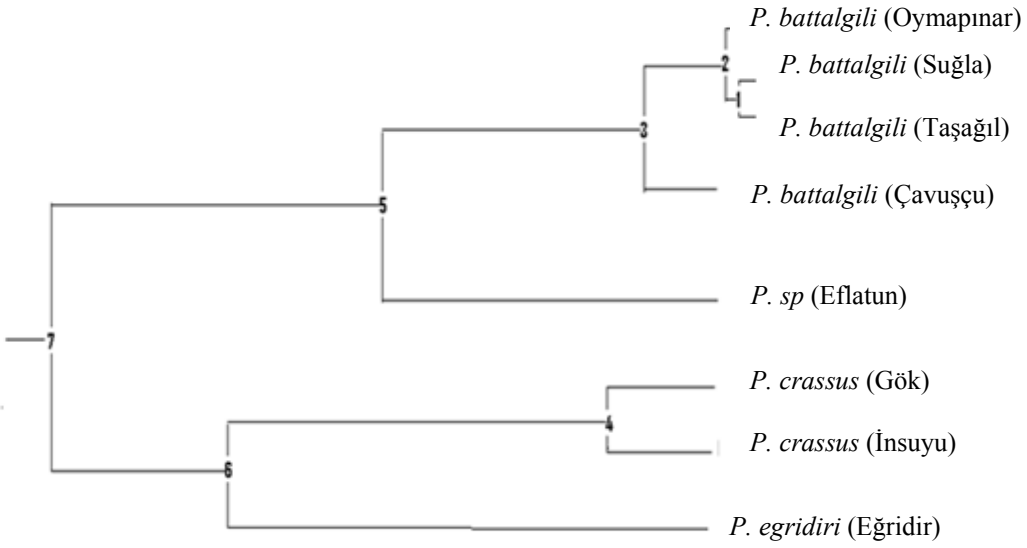

Figure 3.13: UPGMA dendrogram Based Nei's (1972) genetic distance derived from microsatellite data.

3.3 Allozymes

10 allozymes loci in 7 enzyme systems were examined in the study (Table 1.1). 39 alleles were encountered in total. Allele frequency and percentage of polymorphic loci for all populations are shown in Table 3.16. Populations vary in the percentage of polymorphic loci from 10 % to 70 %. In Lake Gök population of *P. crassus* there is only one polymorphic locus (PGD) that has common allele with the frequency of 0.87. *P. egridiri* have 3 polymorphic loci (PGM, PGI-I and PGD) but two of them (PGM and PGD) have common alleles exceeding the frequency of 0.98 and 0.86 respectively. Thus percentage of polymorphic loci for *P. egridiri* can be considered as only 20 % according to 95 % allele frequency criteria.

Of the 10 loci, PGM and PGD have the highest number of allele (6 alleles) and also they have the highest variability among loci. GPI I and II have less variability than PGM and PGD. The other polymorphic loci ICDH, MDH I and II, LDH I have the common alleles exceeding the frequency of 0.96, 0.97, 0.96 and 0.84 respectively. Indeed these loci are monomorphic for most of the populations studied. No allelic variation could be detected at GPDH and LDH II loci for all populations (Table 3.16).

Expected and observed heterozygosity were computed for each locus as well as the observed and expected values for average heterozygosity (Nei's 1973). The values of observed and expected heterozygosity and homozygosity per locus and population and also their mean values with standard deviations were given in Table 3.17. The mean observed heterozygosity ranged from 0.01 (observed in *P. crassus* - both İnsuyu and Gök populations and *P. battalgili* - Lake Suğla population) to 0.08 (observed in *P. sp* - Eflatun population and *P. battalgili* - Lake Çavuşçu Populations). The expected heterozygosity (considered as genetic diversity) ranged from 0.028 to 0.164 for the populations.

Table 3.16: Estimated allele frequencies and percentage of polymorphic loci (% P) in 9 populations of *Pseudophoxinus*.

Locus	Allele	Oymapınar	Gök	Suğla	Eğridir	Insuyu	Taşağıl	Çavuşçu	Eflatun
ICDH	**A**	0.9697	1	1	1	1	0,9655	1	0,9583
	B						0,0345		
	C	0.0303							0,0417
MDH-1	**A**	1	1	1	1	1	1	1	0,9792
	B								0,0208
MDH-2	**C**		1			1		1	
	D						0,0345		
	E	1		1			0,9655		0,9583
	H				1				0,0417
GPI-I	**A**	0.0909		0,0278	0,4848				
	B				0,1818				
	C				0,0758	0,0147	0,0172		0,1458
	D	0.7576	1	0,9722	0,2576	0,9853	0,9828	1	0,8542
	E	0.1515							
GPI-II	**A**							0,1757	
	B							0,1622	
	C	1	1	0,9861	1	1	0,8621	0,6216	0,7917
	D			0,0139			0,1379		0,0417
	E								0,1250
PGM	**A**	0.0152				0,0735			
	B				0,0152				
	C	0.2121	1	0,0417		0,9265	0,1034		0,0417
	D				0,9848				
	E	0.7727		0,9444			0,8103	0,7838	0,6250
	F			0,0139			0,0172	0,1892	0,2500
PGD	**A**	0.0303				0,0294			
	B	0.8030	0,8714	0,9028		0,9412	0,9828	0,7162	0,8542
	C				0,8636				
	D	0.1667	0,1286	0,0972		0,0294	0,0172	0,2027	0,0208
	E				0,1364				
	F							0,0541	
GPDH	**A**	1	1	1		1	1	1	1
	B				1				
LDH-1	**A**	0.8485	1	1		1	1	1	1
	B	0.1515							
	C				1				
LDH-2	**A**				1				
	B	1		1			1	1	1
	C		1			1			
% P		**50**	**10**	**40**	**30**	**30**	**60**	**30**	**70**

Table 3.17: Expected homozygosity and heterozygosity for all loci in 9 populations of *Pseudophoxinus*. (*Expected homozygosity and heterozygosity were computed using Levene (1949). ** Nei's (1973) expected heterozygosity).

P.battalgili (Oymapınar)	**Sample size**	**Obs-Hom**	**Obs-Het**	**Exp-Hom***	**Exp-Het***	**Nei****	**Ave-Het**
ICDH-1	33	1.0000	0.0000	0,9403	0,0597	0,0588	0,0257
MDH-1	33	1.0000	0.0000	1.0000	0.0000	0.0000	0.0051
MDH-2	33	1.0000	0.0000	1.0000	0.0000	0.0000	0.0183
GPI-I	33	1.0000	0.0000	0,5991	0.4009	0.3949	0.1776
GPI-II	33	1.0000	0.0000	1.0000	0.0000	0.0000	0.1481
PGM	33	0.9091	0.0909	0.6368	0.3632	0.3577	0.2314
PGD	33	0.8485	0.1515	0.6685	0.3315	0.3264	0.2266
GPDH	33	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
LDH-1	33	1.0000	0.0000	0.7389	0.2611	0.2571	0.0321
LDH-2	33	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
Mean	33	**0.9758**	**0.0242**	**0.8584**	**0.1416**	**0.1395**	**0.0865**
STDV		**0.0531**	**0.0531**	**0.1744**	**0.1744**	**0.1717**	**0.0975**

P.battalgili (Suğla)	**Sample size**	**Obs-Hom**	**Obs-Het**	**Exp-Hom***	**Exp-Het***	**Nei****	**Ave-Het**
ICDH-1	36	1.0000	0.0000	1.0000	0.0000	0.0000	0.0257
MDH-1	36	1.0000	0.0000	1.0000	0.0000	0.0000	0.0051
MDH-2	36	1.0000	0.0000	1.0000	0.0000	0.0000	0.0183
GPI-I	36	1.0000	0.0000	0.9452	0.0548	0.0540	0.1776
GPI-II	36	0.9722	0.0278	0.9722	0.0278	0.0274	0.1481
PGM	36	0.8889	0.1111	0.8924	0.1076	0.1061	0.2314
PGD	36	0.9722	0.0278	0.8220	0.1780	0.1755	0.2266
GPDH	36	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
LDH-1	36	1.0000	0.0000	1.0000	0.0000	0.0000	0.0321
LDH-2	36	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
Mean	36	**0.9833**	**0.0167**	**0.9632**	**0.0368**	**0.0363**	**0.0865**
STDV		**0.0351**	**0.0351**	**0.0610**	**0.0610**	**0.0601**	**0.0975**

Table 3.17 continued

P.battalgili (Taşağıl)	Sample size	Obs-Hom	Obs-Het	Exp-Hom*	Exp-Het*	Nei**	Ave-Het
ICDH-1	29	1.0000	0.0000	0.9322	0.0678	0.0666	0.0257
MDH-1	29	1.0000	0.0000	1.0000	0.0000	0.0000	0.0051
MDH-2	29	1.0000	0.0000	0.9322	0.0678	0.0666	0.0183
GPI-I	29	0.9655	0.0345	0.9655	0.0345	0.0339	0.1776
GPI-II	29	0.7241	0.2759	0.7580	0.2420	0.2378	0.1481
PGM	29	0.7931	0.2069	0.6642	0.3358	0.3300	0.2314
PGD	29	0.9655	0.0345	0.9655	0.0345	0.0339	0.2266
GPDH	29	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
LDH-1	29	1.0000	0.0000	1.0000	0.0000	0.0000	0.0321
LDH-2	29	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
Mean	29	**0.9448**	**0.0552**	**0.9218**	**0.0782**	**0.0769**	**0.0865**
STDV		**0.1005**	**0.1005**	**0.1163**	**0.1163**	**0.1143**	**0.0975**

P.battalgili (Çavuşçu)	Sample size	Obs-Hom	Obs-Het	Exp-Hom*	Exp-Het*	Nei**	Ave-Het
ICDH-1	37	1.0000	0.0000	1.0000	0.0000	0.0000	0.0257
MDH-1	37	1.0000	0.0000	1.0000	0.0000	0.0000	0.0051
MDH-2	37	1.0000	0.0000	1.0000	0.0000	0.0000	0.0183
GPI-I	37	1.0000	0.0000	1.0000	0.0000	0.0000	0.1776
GPI-II	37	0.6757	0.3243	0.4276	0.5724	0.5646	0.1481
PGM	37	0.8108	0.1892	0.6457	0.3543	0.3495	0.2314
PGD	37	0.7838	0.2162	0.5513	0.4487	0.4427	0.2266
GPDH	37	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
LDH-1	37	1.0000	0.0000	1.0000	0.0000	0.0000	0.0321
LDH-2	37	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
Mean	37	**0.9270**	**0.0730**	**0.8625**	**0.1375**	**0.1357**	**0.0865**
STDV		**0.1222**	**0.1222**	**0.2274**	**0.2274**	**0.2243**	**0.0975**

Table 3.17 continued

P.crassus (Gök)	Sample size	Obs-Hom	Obs-Het	Exp-Hom*	Exp-Het*	Nei**	Ave-Het
ICDH-1	35	1.0000	0.0000	1.0000	0.0000	0.0000	0,0257
MDH-1	35	1.0000	0.0000	1.0000	0.0000	0.0000	0.0051
MDH-2	35	1.0000	0.0000	1.0000	0.0000	0.0000	0.0183
GPI-I	35	1.0000	0.0000	1.0000	0.0000	0.0000	0.1776
GPI-II	35	1.0000	0.0000	1.0000	0.0000	0.0000	0.1481
PGM	35	1.0000	0.0000	1.0000	0.0000	0.0000	0.2314
PGD	35	0.8571	0.1429	0.7727	0.2273	0.2241	0.2266
GPDH	35	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
LDH-1	35	1.0000	0.0000	1.0000	0.0000	0.0000	0.0321
LDH-2	35	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
Mean	35	**0.9857**	**0.0143**	**0.9773**	**0.0227**	**0.0224**	**0.0865**
STDV		**0.0452**	**0.0452**	**0.0719**	**0.0719**	**0.0709**	**0.0975**

P.crassus (İnsuyu)	*Sample size*	*Obs-Hom*	*Obs-Het*	*Exp-Hom**	*Exp-Het**	*Nei***	*Ave-Het*
ICDH-1	34	1.0000	0.0000	1.0000	0.0000	0.0000	0.0257
MDH-1	34	1.0000	0.0000	1.0000	0.0000	0.0000	0.0051
MDH-2	34	1.0000	0.0000	1.0000	0.0000	0.0000	0.0183
GPI-I	34	0.9706	0.0294	0.9706	0.0294	0.0290	0.1776
GPI-II	34	1.0000	0.0000	1.0000	0.0000	0.0000	0.1481
PGM	34	0.9118	0.0882	0.8617	0.1383	0.1362	0.2314
PGD	34	0.9412	0.0588	0.8859	0.1141	0.1125	0.2266
GPDH	34	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
LDH-1	34	1.0000	0.0000	1.0000	0.0000	0.0000	0.0321
LDH-2	34	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
Mean	34	**0.9824**	**0.0176**	**0.9718**	**0.0282**	**0.0278**	**0.0865**
STDV		**0.0316**	**0.0316**	**0.0528**	**0.0528**	**0.0520**	**0.0975**

Table 3.17 continued

P.egridiri (Eğridir)	Sample size	Obs-Hom	Obs-Het	Exp-Hom*	Exp-Het*	Nei**	Ave-Het
ICDH-1	33	1.0000	0.0000	1.0000	0.0000	0.0000	0.0257
MDH-1	33	1.0000	0.0000	1.0000	0.0000	0.0000	0.0051
MDH-2	33	1.0000	0.0000	1.0000	0.0000	0.0000	0.0183
GPI-I	33	0.8485	0.1515	0.3301	0.6699	0.6598	0.1776
GPI-II	33	1.0000	0.0000	1.0000	0.0000	0.0000	0.1481
PGM	33	0.9697	0.0303	0.9697	0.0303	0.0298	0.2314
PGD	33	0.9091	0.0909	0.7608	0.2392	0.2355	0.2266
GPDH	33	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
LDH-1	33	1.0000	0.0000	1.0000	0.0000	0.0000	0.0321
LDH-2	33	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
Mean	33	**0.9727**	**0.0273**	**0.9061**	**0.0939**	**0.0925**	**0.0865**
STDV		**0.0524**	**0.0524**	**0.2157**	**0.2157**	**0.2124**	**0.0975**

(P. sp Eflatun Pınarı)	Sample size	Obs-Hom	Obs-Het	Exp-Hom*	Exp-Het*	Nei**	Ave-Het
ICDH-1	24	1.0000	0.0000	0.9184	0.0816	0.0799	0.0257
MDH-1	24	0.9583	0.0417	0.9583	0.0417	0.0408	0.0051
MDH-2	24	1.0000	0.0000	0.9184	0.0816	0.0799	0.0183
GPI-I	24	0.7083	0.2917	0.7456	0.2544	0.2491	0.1776
GPI-II	24	0.8750	0.1250	0.6374	0.3626	0.3550	0.1481
PGM	24	0.7500	0.2500	0.4468	0.5532	0.5417	0.2314
PGD	24	0.8333	0.1667	0.7323	0.2677	0.2622	0.2266
GPDH	24	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
LDH-1	24	1.0000	0.0000	1.0000	0.0000	0.0000	0.0321
LDH-2	24	1.0000	0.0000	1.0000	0.0000	0.0000	0.0000
Mean	24	**0.9125**	**0.0875**	**0.8357**	**0.1643**	**0.1609**	**0.0865**
STDV		**0.1136**	**0.1136**	**0.1883**	**0.1883**	**0.1844**	**0.0975**

Inbreeding coefficient of an individual relative to the subpopulation is called as Fixation indices (F_{is}) were calculated using only polymorphic loci per locus and populations. F_{is} values are given in Table 3.18. Negative values indicate heterozygote excess and

positive values indicate heterozygote deficiency. The mean F_{is} values ranged from 0.2823 to 8262 is significantly different than zero ($P < 0.01$) indicating heterozygote deficiency for all the populations.

Table 3.18: Fixation index (F_{is}) per loci and populations, Nei (1987)

	Fixation Index (Fis)							
Locus / Pop.	Oymapınar	Gök	Lake Suğla	Lake Eğridir	Insuyu River	Taşağıl River	Lake Çavuşçu	Eflatun River
ICDH	1.0000	-	-	-	-	1.000	-	1.0000
MDH-1	-	-	-	-	-	-	-	**-0.021**
MDH-2	-	-	-	-	-	1.000	-	1.0000
GPI-I	1.0000	-	1.000	0.7704	**-0.0149**	**-0.0175**	-	**-0.171**
GPI-II	-	-	**-0.0141**	-	-	**-0.1600**	0.4256	0.6479
PGM	0.7458	-	**-0.0473**	**-0.0154**	0.3524	0.3730	0.4587	0.5385
PGD	0.5359	0.3625	0.8418	0.6140	0.4769	**-0.0175**	0.5116	0.3642
GPDH	-	-	-	-	-	-	-	-
LDH-1	1.0000	-	-	-	-	-	-	-
LDH-2	-	-	-	-	-	-	-	-
MEAN	**0.8262**	**0.3625**	**0.5409**	**0.7052**	**0.3645**	**0.2823**	**0.4622**	**0.4560**

An UPGMA dendogram and original measures of genetic identity and distance matrix constructed using Nei's (1972) genetic distance are given in Figure 3. 14 and Table 3.19 respectively. The UPGMA dendogram highlights the close genetic similarity of *P. crassus* samples from İnsuyu River and Lake Gök and also *P. battalgili* from Lake Suğla and Taşağıl River. *P. egridiri* population has the highest genetic distance from the other populations due to unique allele observed in PGM, PGD, LDH I and II loci for *P. egiridiri* population. *P. battalgili* population from Lake Çavuşçu has higher genetic distance than *P. sp* to the other *P. battalgili* populations (Figure 3.14).

Table 3.19: Nei's (1972) original measures of genetic identity and genetic distance.

	Oymapınar	**Gök**	**Suğla**	**Eğridir**	**Insuyu**	**Taşağıl**	**Çavuşçu**	**Eflatun**
Oymapınar	---	0.7097	0.9909	0.3631	0.7132	0.9870	0.9754	0.9810
Gök	0.3429	---	0.7005	0.3459	0.9986	0.7129	0.6823	0.7035
Suğla	**0.0092**	0.3560	---	0.3475	0.7061	0.9963	0.9855	0.9870
Eğridir	1.0130	1.0617	1.0569	---	0.3465	0.3367	0.3251	0.3392
Insuyu	0.3379	**0.0014**	0.3479	1.0598	---	0.7196	0.6858	0.7103
Taşağıl	**0.0131**	0.3384	**0.0037**	1.0885	0.3290	---	0.9847	0.9903
Çavuşçu	**0.0249**	0.3823	**0.0146**	1.1236	0.3771	**0.0154**	---	0.9861
Eflatun	**0.0191**	0.3517	**0.0130**	1.0811	0.3420	**0.0097**	**0.0140**	---

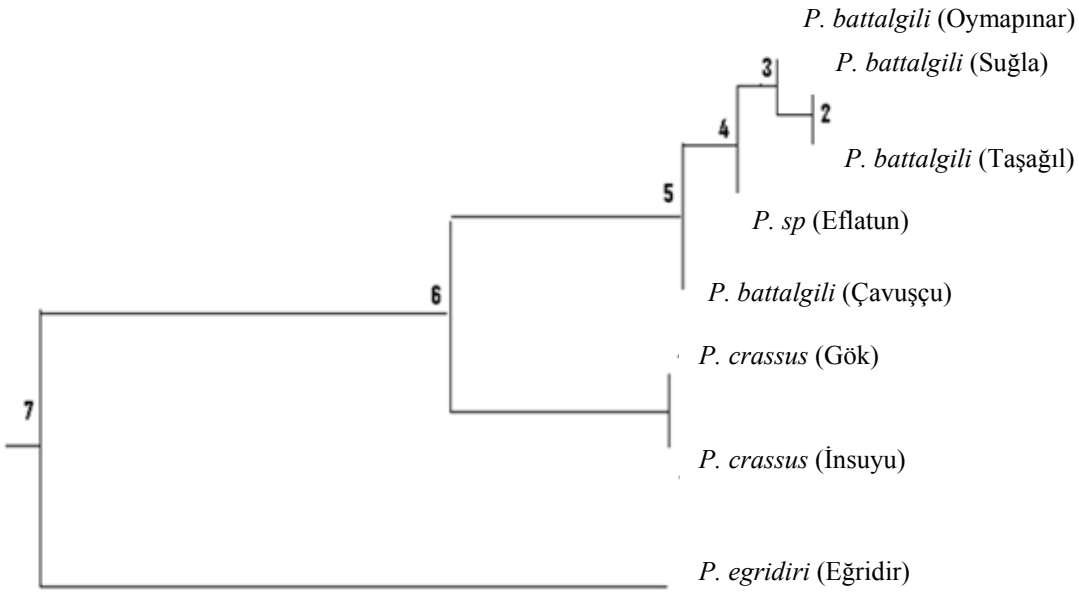

Figure 3.14: UPGMA dendrogram based on Nei's (1972) genetic distance derived from allozyme data (Popgen32).

3.4 The relationship between genetics and morphological variation

3.4.1. Microsatellites vs Morphology

In order to assess the relationship between morphological and genetic variance, procrustes distances of each individual were correlated with individual heterozygosity. Individual heterozygosity was calculated as the number of loci at which the individual was heterozygous, divided by the total number of loci at which an individual was scored.

Figure 3.15 show the relationship between Procrustes Distance (d^2) and mean individual observed heterozygosity derived from microsatellite data. Procrustes Distances is used as shape variation of each individual from the mean shape of the population. Mean shape were calculated for each population separately. Statistically significant negative correlations were observed in *P. battalgili* of Lake Suğla and Lake Çavuşçu , *P. crassus* of Lake Gök and *P. sp* of Eflatun River populations at α=0,05 level. *P. battalgili* of Seydisehir population has also significant negative correlation at α=0,1 level. P. battalgili of Oymapınar population has weak correlation between morphological and genetic variances that is significant at α=0,25 level. *P. egridiri* and *P. crassus* of İnsuyu populations have an weak positive correlation.

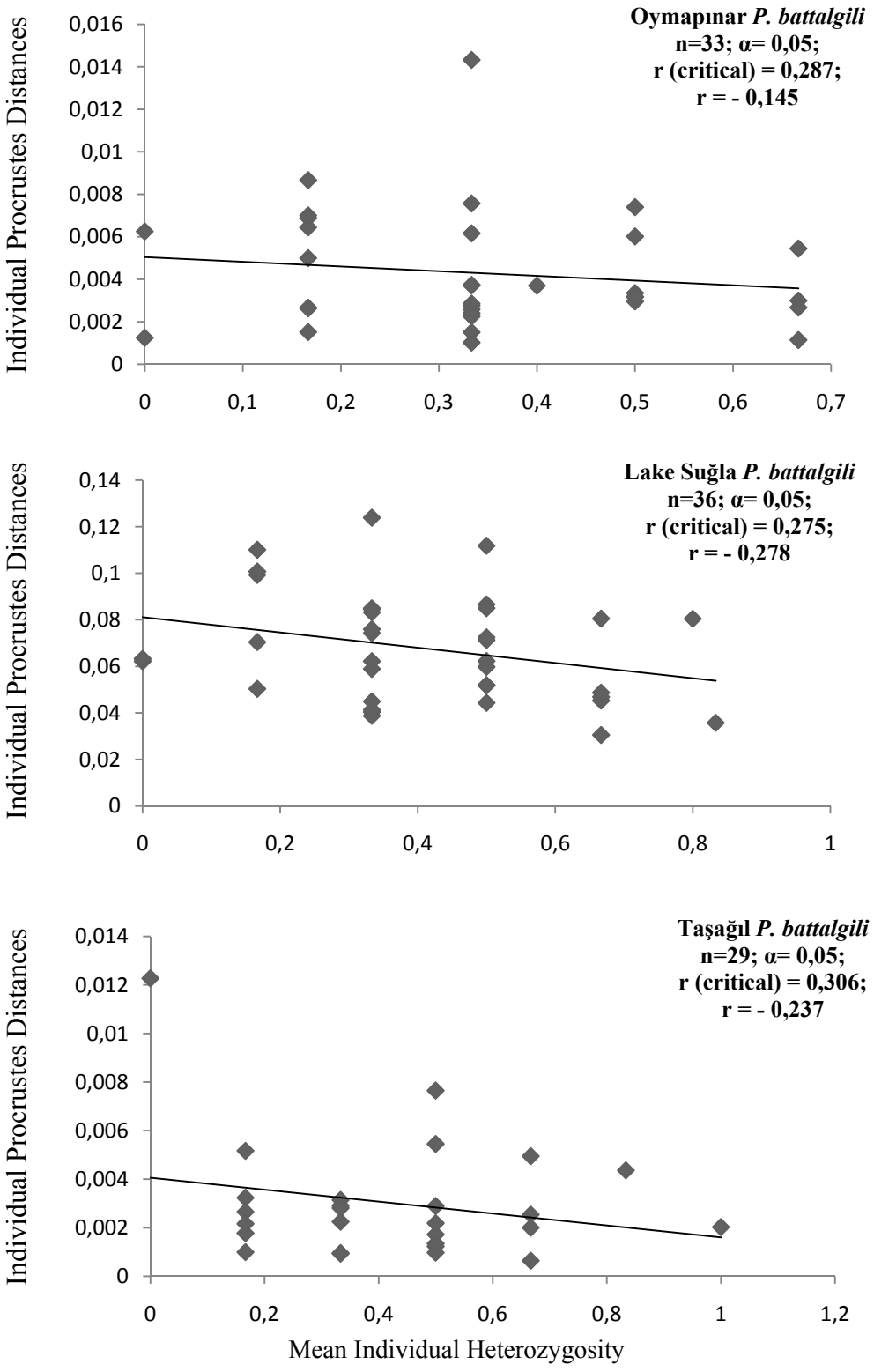

Figure 3.15: The relationship between Morphological Variation (Procrustes Distance) and mean individual heterozygosity derived from microsatellite data.

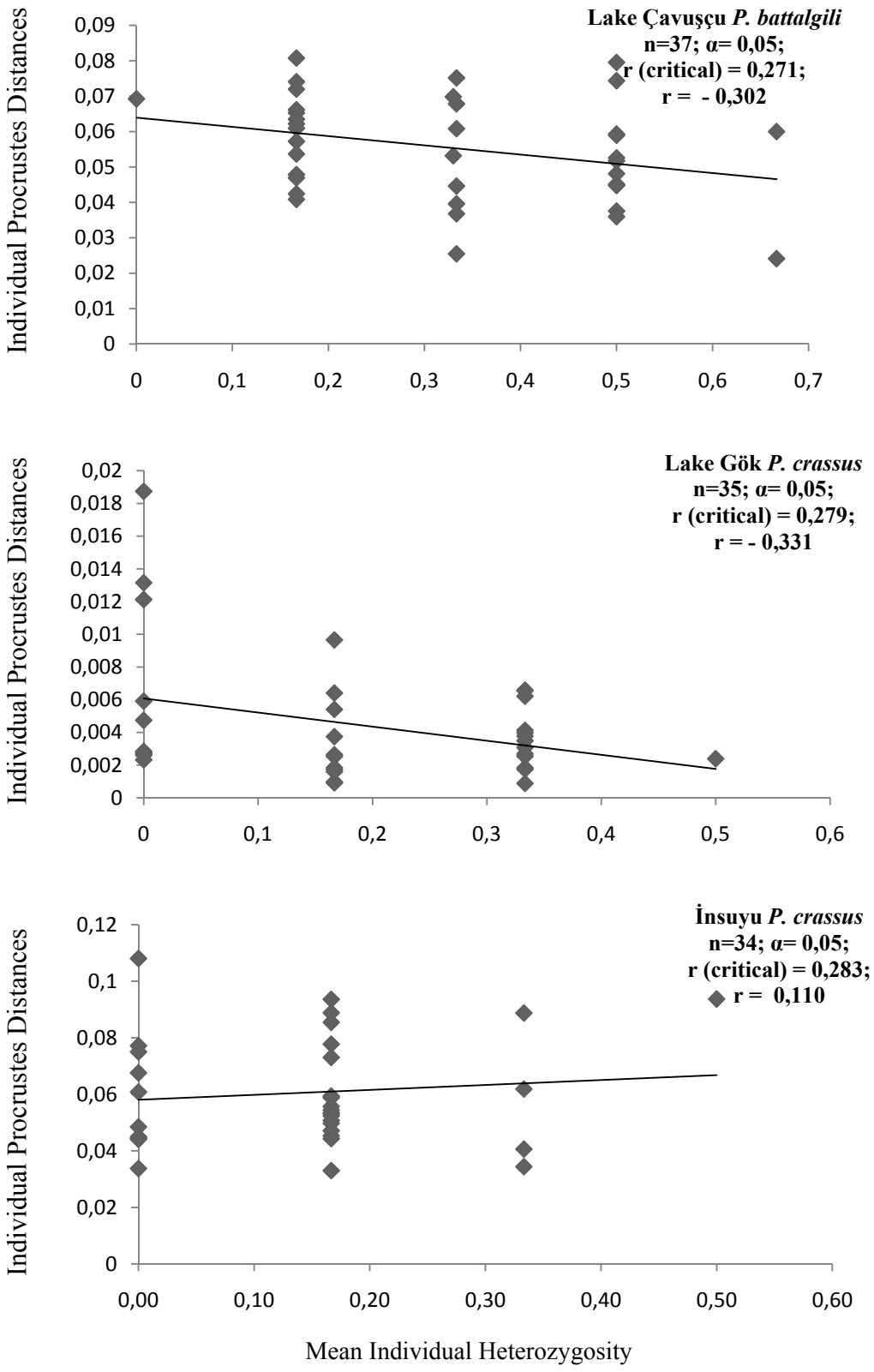

Figure 3.15: Continued

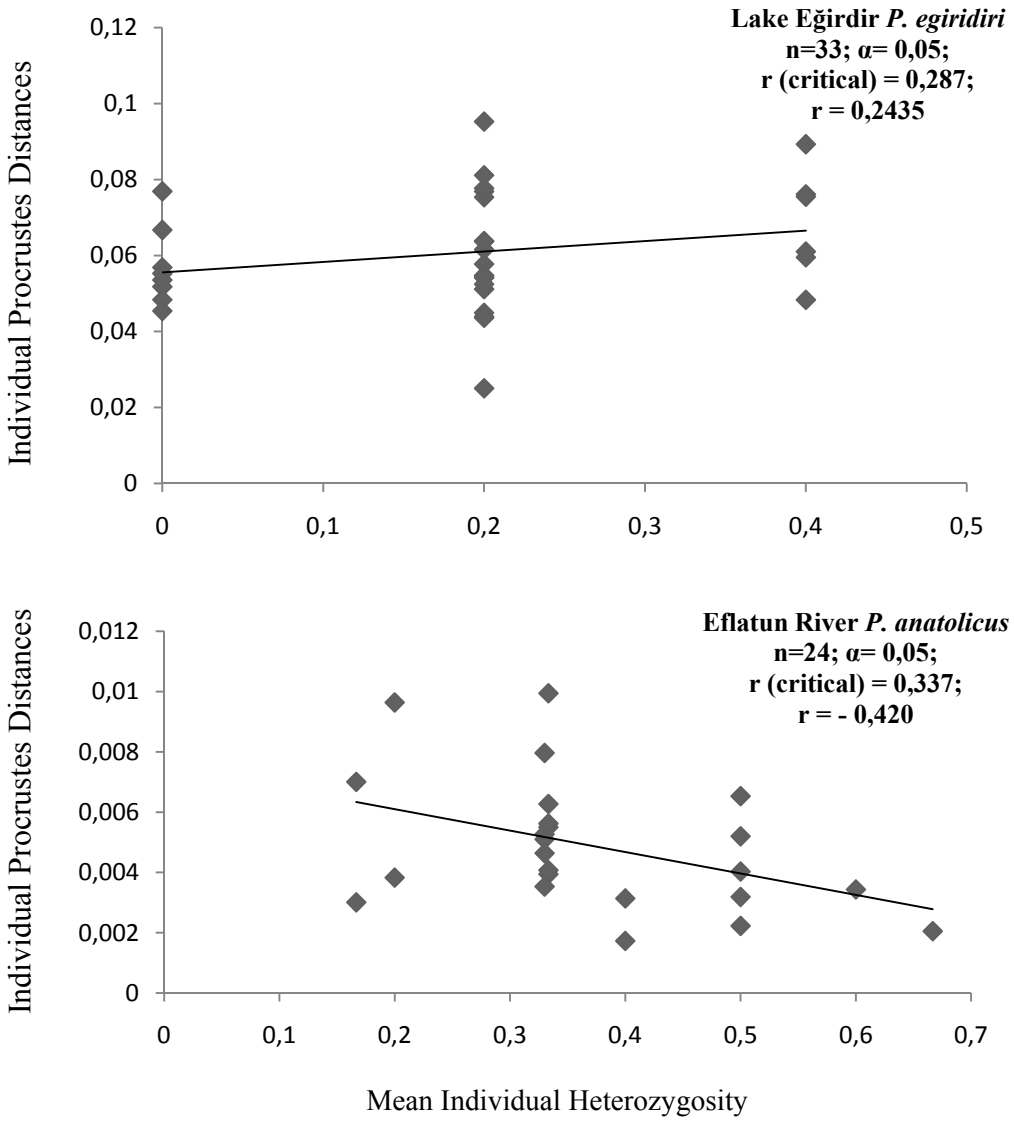

Figure 3.15: Continued

A strong statistically significant negative correlation was observed between the mean individual heterozygosity and morphological variation (Procrustes distances) for the data pooled from eight *Pseudophoxinus* populations (Figure 3.16).

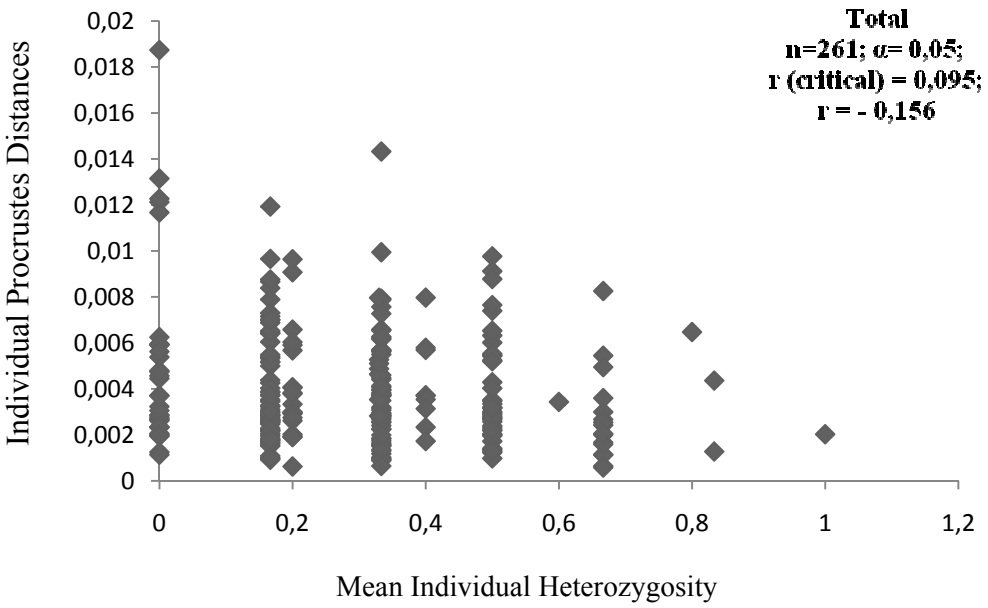

Figure 3.16: The relationship between mean individual heterozygosity derived from microsatellite data and morphological variation (Procrustes distances) for the data pooled from eight *Pseudophoxinus* populations.

Figure 3.17 show the relationship between mean morphological variance and mean percentage of observed heterozygosity at population level. There is intermediate negative relationship was observed (r = - 0,139 and $r_{critical}$ = 0,549). The relationship between inbreeding coefficient (or Fixation index, F_{is}) and mean procrustes distance at population level is given in Figure 3.18. There is an intermediately significant positive relationship was observed at α = 0,05 level, and a significant correlation at α = 0,25 level ($r_{critical}$ = 242; df=7; $r_{observed}$ = 263).

Moreover, combining probabilities (P values) of correlation coefficient from significant test show that there is a statistically significant negative correlation between heterozygosity and morphological variance (P < 0,001). That test is very useful method to combine different sets of data testing the same scientific hypothesis.

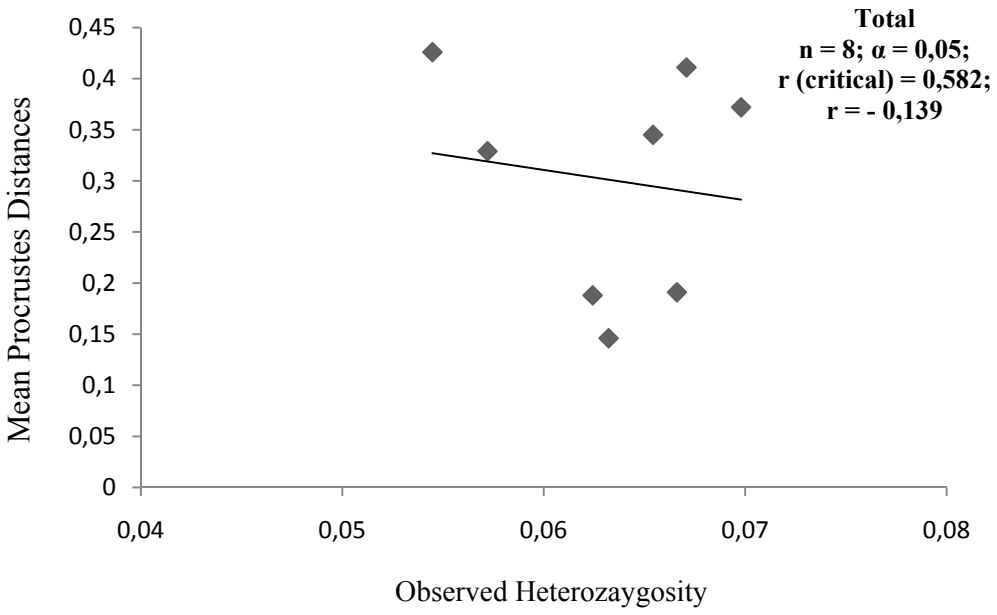

Figure 3.17: The relationship mean procrustes distances and observed mean heterozygosity for eight *Pseudophoxinus* populations.

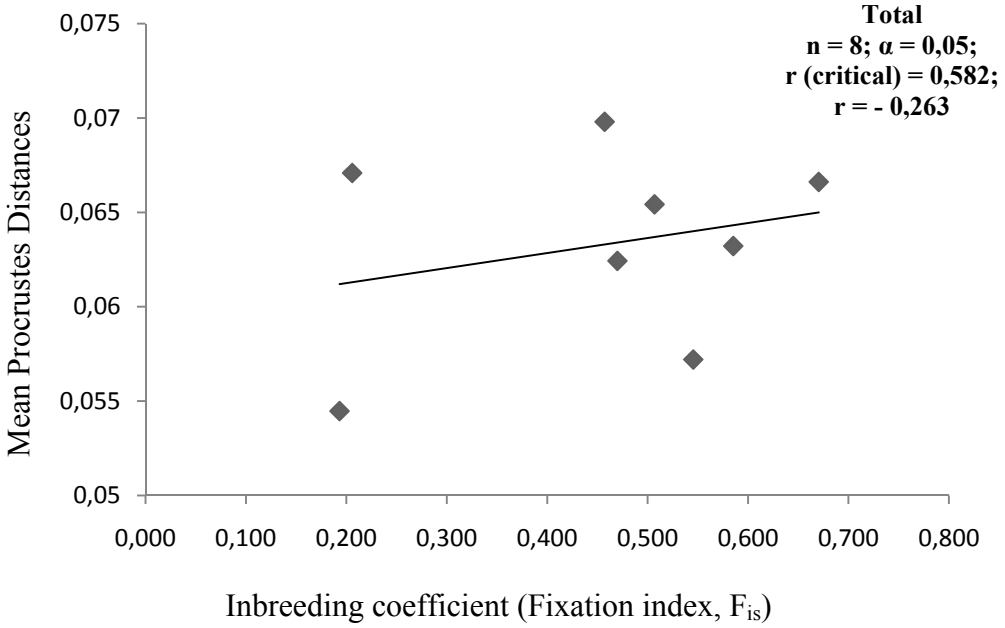

Figure: 3.18: The relationships between mean procrustes distances and inbreeding coefficients (F_{is}) for eight *Pseudophoxinus* populations.

3.4.2. Allozyme vs Morphology

Figure 3.19 show the relationship between procrustes distance and mean individual observed heterozygosity derived from allozyme data. There is no any statistically significant correlation observed between individual mean allozyme heterozygosity and shape variation within populations. Only *P. egridiri* show weak negative correlation. *P. sp* population has a weak positive tendency. There is also no statistically significant relationship observed between shape variation and allozyme heterozygosity when all data are pooled from eight *Pseudophoxinus* populations (Figure 3.19)

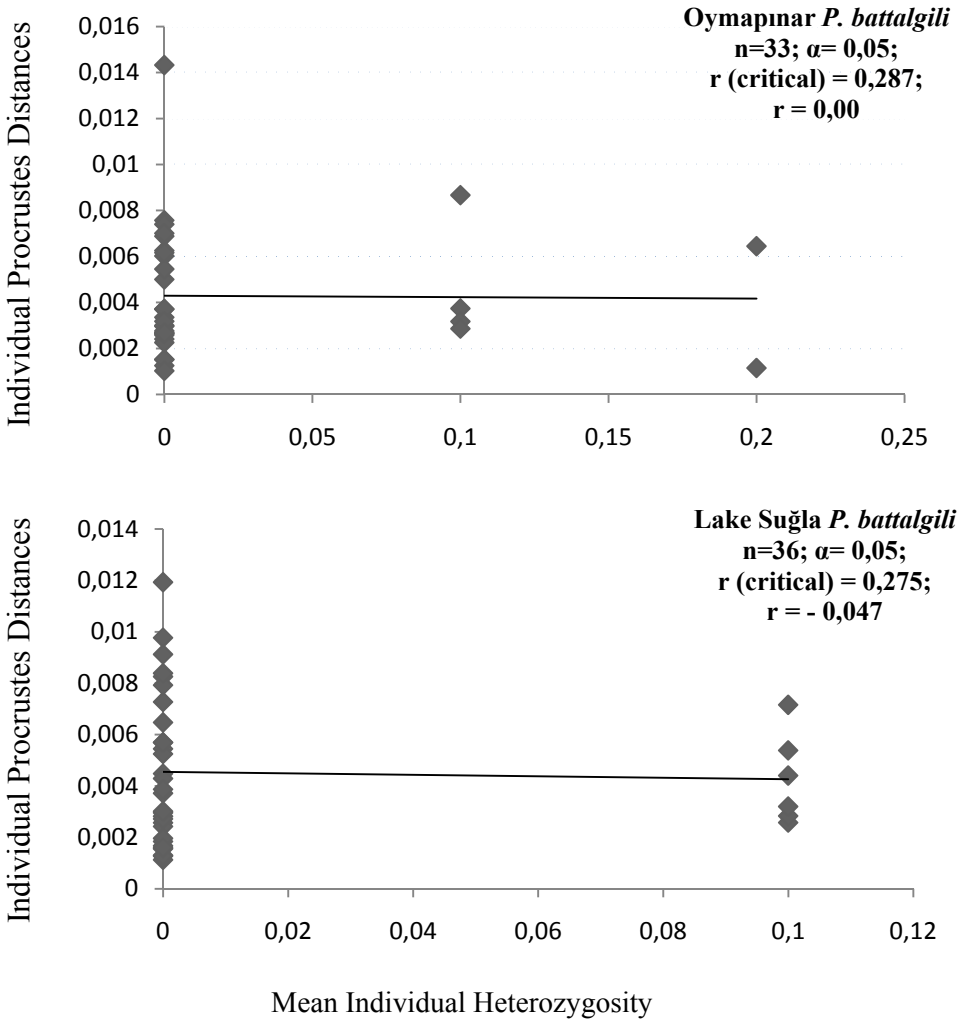

Figure 3.19: The relationship between Procrustes Distance and mean individual observed heterozygosity derived from allozyme data.

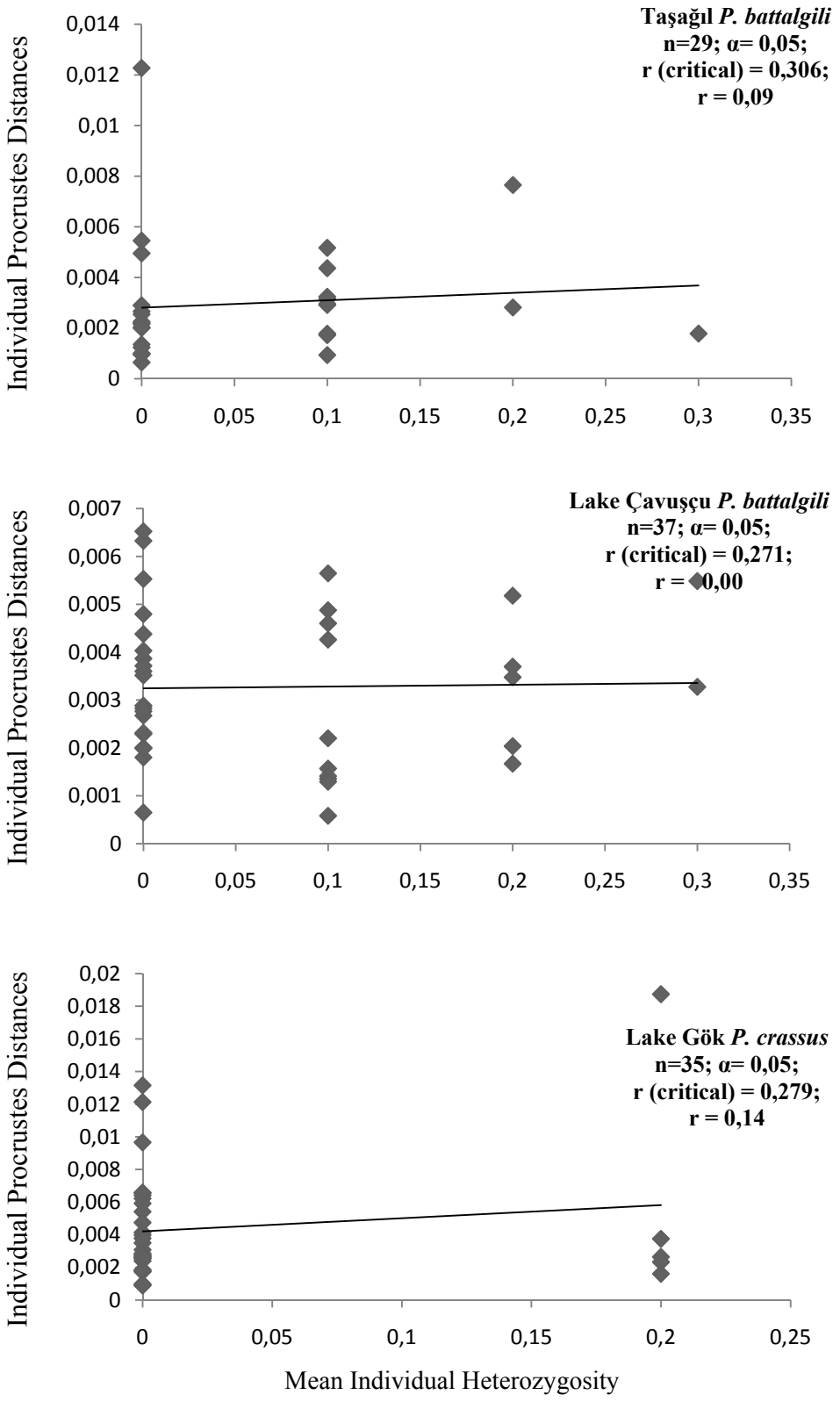

Figure 3.19: Continued

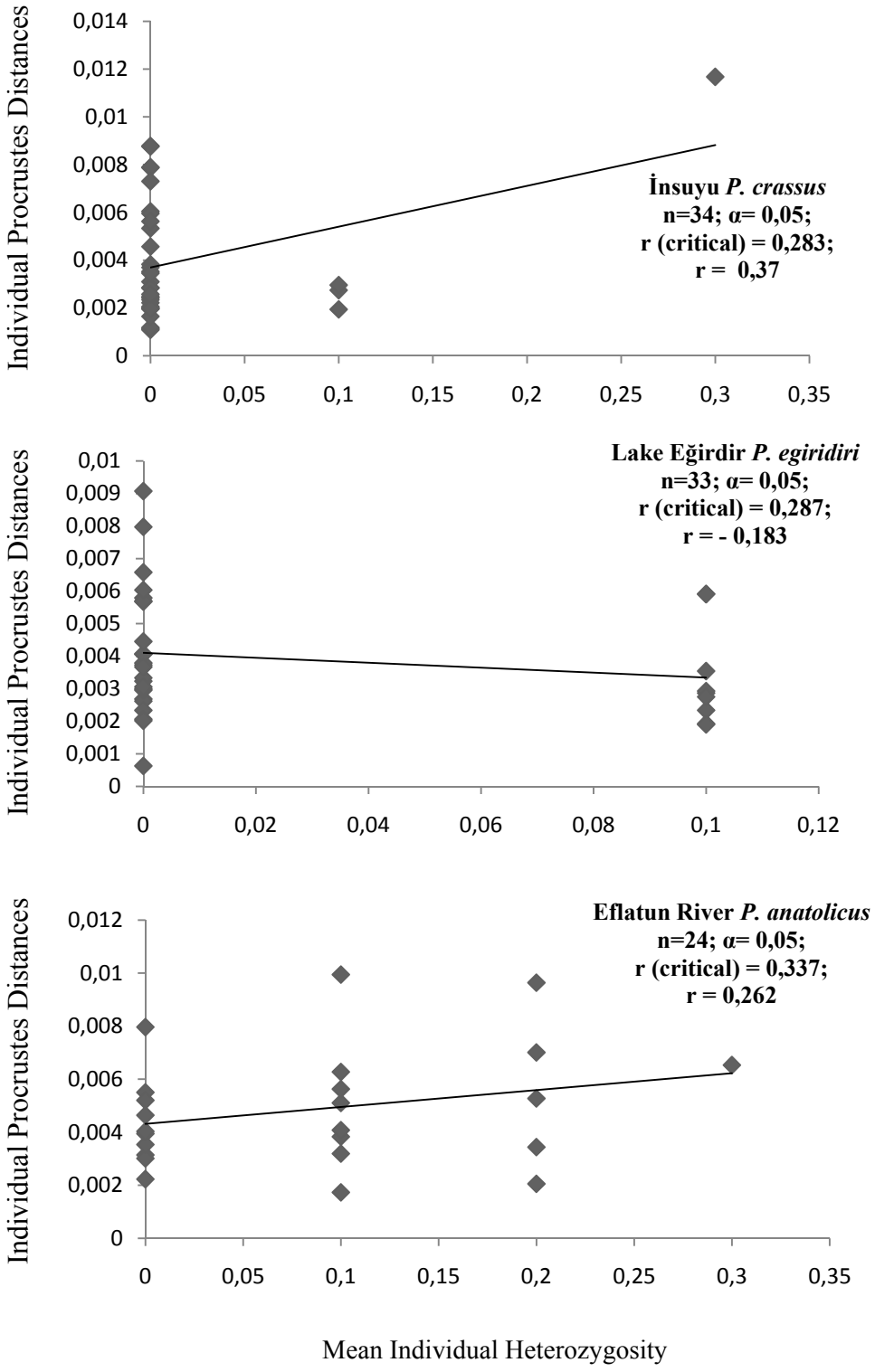

Figure 3.19: Continued

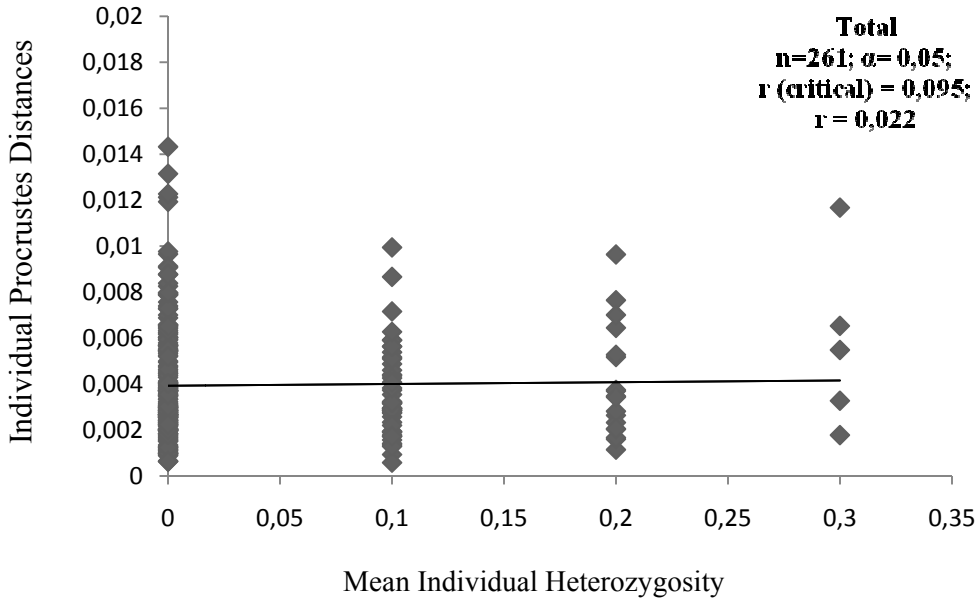

Figure 3.20: The relationship between mean individual heterozygosity derived from microsatellite data and morphological variation (Procrustes distances) for the data pooled from eight *Pseudophoxinus* populations.

Figure 3.21 shows the relationship between mean procrustes distance and mean percentage of observed heterozygosity derived from allozyme data at population level. There is intermediate weak relationship was observed between two variables.

Figure 3.22 shows the relationship between inbreeding coefficient (or Fixation index, F_{is}) and mean procrustes distance at population level. An intermediately statistical significant positive relationship was observed at $\alpha = 0,05$ level, but it is significant at $\alpha = 0,25$ level ($r_{critical} = 242$; df=7; $r_{observed} = 310$). Morphologic variation seems to be increased with increasing inbreeding coefficient.

Combining probabilities (P values) of correlation coefficient from significant test did not show any tendency between heterozygosity and morphological variance (P > 0, 25).

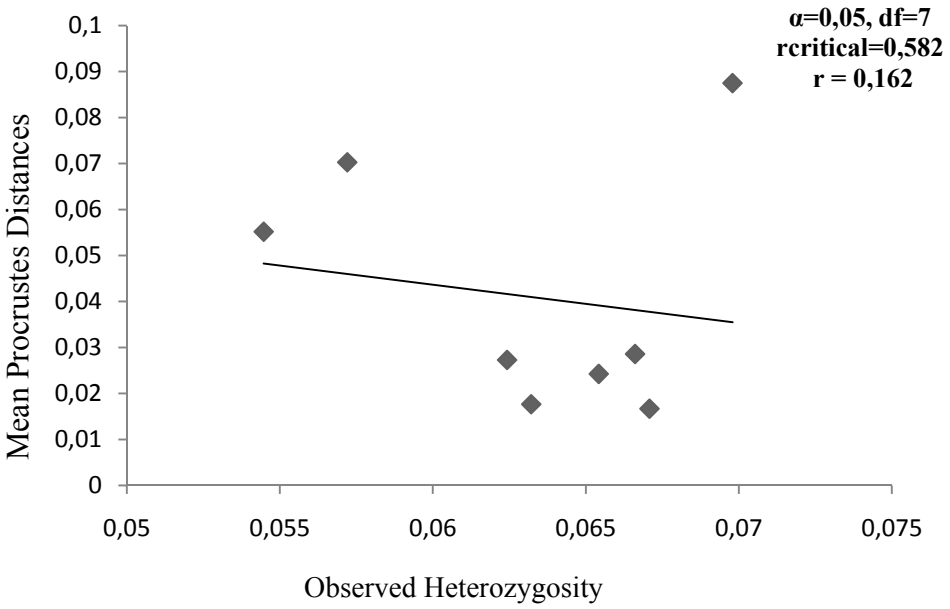

Figure 3.21: The relationship mean procrustes distances and observed mean heterozygosity derived from allozyme data for eight *Pseudophoxinus* populations.

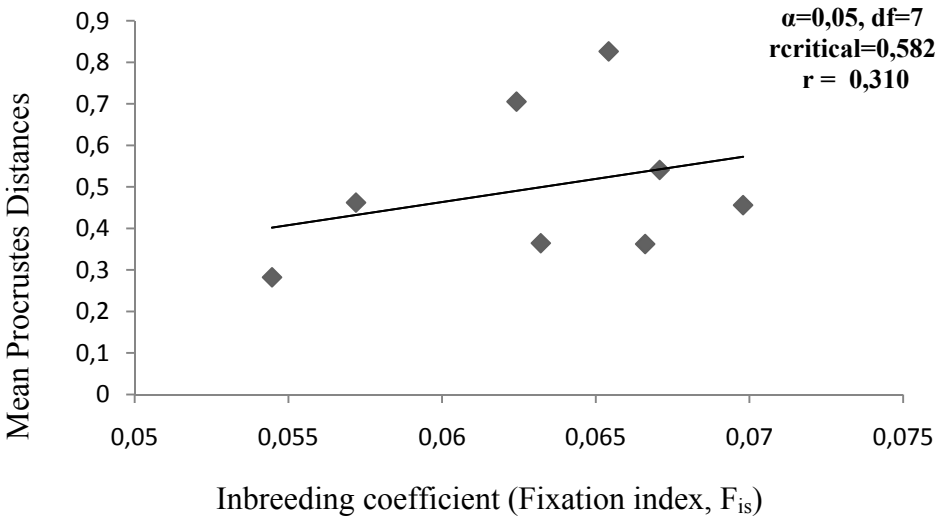

Figure: 3.22: The relationships between mean procrustes distances and inbreeding coefficients (F_{is}) derived from allozyme data for eight *Pseudophoxinus* populations.

CHAPTER 4

DISCUSSION

Phylogenic Structure:

Pseudophoxinus genus seems to be a very good candidate to study genetic effects on phenotypic fitness traits such as morphology as they are ecologically flexible cyprinid fishes and show wide range of ecomorphological adaptation in Anatolia such a geographical and ecological diverse environment. However, as the degree of intraspecific divergence among *Pseudophoxinus* lineages in central Anatolia is high, the taxonomy of this genus is still confusing for some of the species (Hrebek 2004). Samples analyzed in the present study include species having a consensus about their classification except for *P. sp* Eflatun River population in the literature (Hrebek 2004). *P. sp* is recorded as *P. battalgili* in the study of Hrebek (2004) based on mitochondrial DNA. In the present study, *P. sp* was clustered with *P. battalgili* population in UPGMA dendogram derived from both microsatellite and allozymes data (Figures 3.13 and 3.14). Moreover, *P.* sp and *P. battalgili* were found as identical groups in the analyses of genetic identity with allozymes data (Table 3.19). Gene flow between Eflatun River and *P. battalgili* populations was estimated more than other populations (Table 3.15). However, Fst values sowed that there is statistically significant genetic differentiation between *P. battalgili* and *P*. sp (Table 3.14). On the other hand, Eflatun River population showed morphologically divergence from *P. battalgili* in geometric morphometry analyses, although landmarks used in the present study were not representative for whole morphological characters. Thus it can be concluded that Eflatun River population seems to be close group of *P. battalgili*, but they are not identical. In order to make a comprehensive interpretation about Eflatun River

population, more species should be analyzed together using both molecular markers and appropriate morphometric characters.

The UPGMA dendogram (Figure 3.4) based on landmark data from head region shows three distinctive groups among the populations. Taşağıl River and Lake Suğla populations of *P. battalgili* were closely associated in the same groups, though Oymapınar and Çavuşçu populations of *P. battalgili* were clustered with *P. egridiri* and İnsuyu population of *P. crassus*. Populations of *P. crassus* (Lake Gök) and *P. sp* (Eflatun River) were clustered in the same group. Ecological factors may act on convergent of the Lake Gök *P. crassus* and Eflatun River *P. sp* populations. Elatun River actually looks like small and isolated pool or wetland area rather than river having strong water current (Personal observation). Thus, adaptation to such condition may lead to a morphological convergence between a lake population *P. crassus* and *P. sp*.

Results in the present study are not coincident with previous studies based on morphological data in the literature (Bogutskaya 1992). These differences may be due to landmarks restricted only to head region of the specimens in the present study that may not be representative for morphological features especially within species located from different environments requiring different ecomorphological adaptations. Head formations of fish species is closely related with ecomorphological adaptation so that populations of the same species located in different environmental conditions may have different phenotypic representation from each other. It is well known fact that fish populations show wide range phenotypic plasticity (Hedenskog *et al*. 1997).

On the other hand UPGMA dendogram based on Nei's (1972) genetic distance derived from both allozyme and microsatellite match each other but they did not support morphological data. *P. crassus* and *P. battalgili* populations do not show divergence within species. Pairwise F_{st} values show very high differentiation between species at 0.05 significant level (Figure 3.13 and 3.14; Table 3.14). *P. egridiri* show the highest divergence from other groups and pairwise F_{st} values ranged from 0.32 to 0.52. It is not

surprising, as *P. egridiri* is considered as to be sister taxon of *Phoxinellus phoxinus*, not a member of clade of other *Pseudophoxinus* species (Hrbek *et al*. 2003).

On the other hand, *P. crassus* populations have a significant differentiation within species at level of 0.09 of F_{st} value (Table 3.18). İnsuyu population has significant divergence form Lake Gök population although they are geographically close populations. Moreover, gene flow among *P. crassus* populations (2.456 Nm values, Table 3.15) was much lower than among *P. battalgili* population (8.1 to 13.9 Nm value, Table 3.15) despite geographic distance between *P. battalgili* populations. High gene flow and genetic convergence between Taşağıl River and Lake Suğla populations of *P. battalgili* is explicable due to existence of river connection between two populations. Taşağıl River population was collected on the river basin away from Lake Suğla around 30 km. Suğla population was collected from incoming mouth of a irrigating cannel located opposite side of the Lake Suğla. There is no geographical barrier between two populations except for the lake itself (Personal observation). However, higher gene flow between Lake Çavuşçu and Oymapınar Dam of *P. battalgili* populations compare to İnsuyu and Lake Gök populations of *P. crassus* is a question mark.

It was observed considerable morphological differentiation between lake and river populations that lake populations have wider and shorter head formation than river populations. River populations seem more elongated and thin than lake forms. Elongated head formations of river populations have been described as characteristics for river habitats with faster water current. So, such body formations can be considered as an ecomorphological adaptation for their surroundings (McGuigan *et al* 2003).

Structure of Population Genetic:

The present study showed that allozyme variation among *Pseudophoxinus* populations was remarkably low. Average heterozygosity is ranged from 0.014 to 0.087 (Table 3.17) and the percentage of polymorphic loci in which alleles do not exceed the frequency of 0.95 is ranged from 10% to 40% (Table 3.16). Differently than allozyme

locus, majority of microsatellite locus are polymorphic for all populations. Observed average heterozygosity (0.14 to 0.46) was much higher than allozymes (Table 3.12).

Both *P. crassus* populations were detected as the least variable population at allozyme diversity among the populations with around 0.01 mean heterozygosity and polymorphic loci not exceeding 20%. Microsatellite results is coincident with allozyme data that both İnsuyu and Gök populations of *P. crassus* and *P. egridiri* seem to be least variable groups as regard with observed heterozygosity (0.14; 0,206 and 0.16 respectively). There is heavy habitat destruction around Lake Gök because of dense agricultural activity and loss of water level. The same situation can be observed for İnsuyu River. As the river has been used for agricultural activity by local people, water level decreases dramatically especially in dry period. Although there is no scientific record about demographic structure of this species, the population density is expected to decrease (personal communication Güler Ekmekçi). So *P. crassus* was recorded as endangered (EN) status in IUCN Red List in 2004.

P. egridiri population has also comparatively very low genetic diversity associated with observed heterozygosity and number of polymorphic locus. Observed heterozygosity was 0.16 for microsatellite and 0.027 for allozymes data. This species was known nearly expired from Lake Eğridir (personal communication Fahrettin Küçük), but a small population is still survival in northern part of the lake. Although, there is no any demographic structure of this population, it is believed that population structure is dramatically declined due to habitat loss and introduced alien predator species (Cirvelli 2006). *P. egridirii* was recorded as critically endangered (CR) conservation status in IUCN Red List 2004. Population genetic data in the present study support the conservation status determination. *P. sp* seems to be the population having highest genetic diversity associated with observed heterozygosity estimated both from microsatellite and allozymes (0.09 and 0.40 respectively). Although, they were sampled from an isolated small pool, there was a gene flow (between 0.5 and 1.6) from all the population studied. This gene flow may contribute genetic diversity of this population.

Gene vs Morphology:

The theory of *genetic homeostasis* by Lerner's (1954) proposed that high individual heterozygosity result in low morphological variance from the population mean because of effective buffering mechanisms of heterosis. Heterosis refers to high level of heterozygosity that buffer the organisms from perturbations affecting developmental process. However the relationship between heterozygosity and morphological variation in the level of population, species or higher taxa seems to be problematic (Zink *et al*. 1985). It has been proposed that if Lerner's hypothesis is true, a negative correlation between heterozygosity and morphological variance should be observed among population or species. On the other hand, if the hypothesis is wrong, morphological and genetic variation can be two operationally independent estimates of total genetic variation and they can be considered as different aspects of the same underlying phenomenon. Thus there should be a positive relationship between these two variations (Strauss 1989).

However, results of empirical studies about correlation between morphological variation and level heterozygosity have shown highly diverse associations from nonexisting to significant both negative and positive relationship (cited in Zink *et al* 1985). Studies of Leary *et al*. (1983) and Leary (1985) showed that individuals having greater heterozygosity screened at around 40 allozyme loci have higher developmental stability in morphological traits of salmonid fishes. They found that phenotypically extreme and asymmetric individuals were homozygote. Shikano *et al*. (2005) found that there is strong negative correlation between both allozyme and microsatellite heterozygosity and verbal deformity in domestic strains of guppy populations. Conversely, a strong positive correlation was found between mean heterozygosity at 20 allozyme loci and morphological variation among 32 samples, representing eight taxa of bottom dwelling freshwater fish sculpins (*Cottus*) (Strauss 1989, 1991).

On the other hand, several studies on *Drosophila* (Fowler and Whitelock 1994; Gilligan *et al*. 2000), rambur (Carchini *et al*., 2001), yellow dung flies (Hosken *et al*., 2000) and

Atlantic Salmon (Gjerde *et al*., 2005) have failed to find any trend between genetic and morphological variations. The common point in these studies is that genetic variation estimations were based on low number of polymorphic allozyme locus (not exceeding 7 locus), or inbreeding depression coefficient using pedigree analyses.

The present study showed that there is statistically significant negative correlation between shape variation and mean individual heterozygosity derived from microsatellite data (Figure 3.15). The same trend was separately observed in population level except for *P. egridirii* and *P. crassus* (İnsuyu) populations. Correlation coefficients observed from the population were analyzed using combining probabilities (P values) of correlation coefficient from significant test. Results show highly statistically significant negative correlation between shape and genetic variations ((P < 0,001). This test is very useful method to combine different sets of data testing the same scientific hypothesis. The same negative correlation was also observed between mean heterozygosity and mean shape variation in population level, although these are not statistically significant (Figure 3.16). Intermediately positive correlation observed between inbreeding coefficient and mean shape variation of populations also support Lerner's theory (Figure 3.17).

However, the comparison of heterozygosity derived from allozyme data and morphological variation did not give any tendency in individual level (Figure 3. 18). Combining probabilities (P values) of correlation coefficient from significant test did not give any significant correlation (P > 0,25). Differently than individual level, mean shape variation showed weak negative correlation with mean heterozygosity (Figure 3.20) and an intermediate positive correlation with inbreeding coefficient (Figure 3.21) in population level.

The theory of genetic homeostasis (Lerner 1954) was supported with microsatellite data in the present study. Only two populations (*P. egridirii* and *P. crassus* -İnsuyu) did not show negative correlation between shape and heterozygosity. Comparatively low number of polymorphic loci and low diversity in mean individual heterozygosity

observed for these populations may prevent to reveal potential relationship between variants. However, allozyme data did not coincident with the Lerner's theory that all the populations did not show any trend in the relationship between shape and heterozygosity variations. Narrow range of heterozygosity and limited number of polymorphic loci may be one of the reasons for nonexisting relationship. That made it very difficult to find any significant correlation. Similarly, most of the previous study performed with low number of polymorphic allozyme loci have also failed to reveal any trend between shape variation and allozyme data (Flowler and Whitelock 1994; Gilligan *et al*. 2000; (Carchini *et al*., 2001; Hosken *et al*., 2000; Gjerde *et al*., 2005).

Consequently, comparison of microsatellite and allozyme data of this study showed that number of polymorphic loci and level of heterozygote diversity might be one of the most important factors in order to reveal possible relationship between shape and genetic variants. As polymorphisms are often uncommon for allozyme, it has been suggested that at least 40 loci should be examined in order to acute estimation of heterozygosity of a population (Sjorgen and Wyoni 1994). For instance, studies with high number of polymorphic allozyme loci generally provide both substantial negative (Leary *et al*. 1983; Leary, 1985; Shikano *et al*. 2005) and positive (Strauss 1989 and 1991) correlations between shape and genetic variations.

On the other hand, the quality of allozyme as a genetic marker to estimate genetic diversity is still arguing because some of it's' disadvantages (e.g. null alleles and low resolution of genotyping) leading to underestimation of genetic diversity (David 1998). Additionally, allele frequency is influenced by natural selection, and the degree of neutrality is still unknown for some of allozyme locus (Clarke 1979). Within this perspective, microsatellite seems to be more relevant marker to estimate genetic diversity and correlate with shape variations within and among populations as they are considered as neutral and much more polymorphic markers than allozymes. So that, the degree of inbreeding coefficient and level of heterozygosity can be more closely calculated by microsatellites than allozymes markers (Britten 1996).

A striking result in the literature associated with relationship between shape and genetic variations is given in the study of Strauss (1989 and 1991). He found a statistically highly significant positive correlation in his study performed with quite number of samples and polymorphic allozyme loci. This finding seems to be completely opposite to the genetic homeostasis and canalization theory. This contradiction can be explained by an adaptive plastic strategy of organisms' phenotype for heterogeneous environment. Evolutionary models dealing with phenotypic plasticity show that phenotypic plasticity of organisms can be favored rather than canalization by natural selection in heterogeneous environmental conditions (Relya 2002). Thus, different adaptive phenotypes can be survived within population in such environmental conditions. Bottom dwelling freshwater fish species investigated in the study of Strauss (1991) have been found much more heterogeneous environment than pelagic fish species. Consequently, in order to fulfill the whole picture of shape response to the genetics and environmental variability, different species from different environmental conditions should be investigated associated with the relationship between shape variation and heterozygosity.

Moreover, methods used in Strauss's study to correlate heterozygosity and morphologic variation might be a reason for this opposite results. In his study morphologic and genetic data were not collected from the same individuals. He has used mean values of heterozygosity and morphological variance derived from different individuals of the same populations. That may cause bias in estimation of correlations.

The other factor that may affect the relationship between shape and genetic variations is the size affects on shape variation. As shape can change with size during process of fish growth, it is very important to test whether shape changes as a function of size to reveal that shape of an individual vary from the population mean with level of individual heterozygosity independently from size. Shape and size analyses by regressing landmark coordinate data on centroid size using TpsReg program showed that shape varied independently from size within population, but this is not the case among species. On the other hand, there were variable results observed among the

populations of the same species. *P. battalgili* have significant variation in shape with size among the populations but there was no significant size affect on shape variation between two *P. crassus* populations. This suggests that shape variation from the population mean can be correlated with heterozygosity independently from size within population.

CHAPTER 5

CONCLUSION

Results of the present study revealed that the theory of *genetic homeostasis* (Lerner 1954) is confirmed with microsatellite data. Thus, it can be concluded that shape variation seems to be negatively correlated with mean individual heterozygosity in *Pseudophoxinus* populations.

However, allozyme data did not reveal any relationship between shape and genetic variability. Comparison of microsatellite and allozyme data in this study showed that the level of polymorphic loci and heterozygosity is one of the most important factors to reveal possible relationship between shape and genetic variants. Thus, microsatellite seems to be more appropriate marker than allozyme in order to reveal genetic variation and its relationship with the shape variation.

Shape of *Pseudophoxinus* varied independently from size within population, but this is not the case among species. This suggests that shape variation can be correlated with heterozygosity independently from size within *Pseudophoxinus* population.

On the other hand, comparison of some of the literature and the present study suggest the idea that organism can show different phenotypic adaptive strategy in their response to different environmental conditions. Fish populations in heterogeneous environmental conditions like bottom dwelling species can show different adaptive phenotypes that may increase shape variation (Relya 2002).

The classification of *Pseudophoxinus* populations constituted by geometric morphometric data did not match the previous study in literature (Bogutskaya 1992). *P. battalgili* and *P. crassus* populations show high divergence within species in UPGMA dendogram based on geometric morphometric data. However, that contradiction is not the case with microsatellite and allozyme UPGMA dendograms based on genetics distance. Eflatun River population seems to be very close to *P. battalgili* population according to genetic distance, but these two groups were not identical associated with Fst values.

Lake and River populations show considerable morphological differentiation. Lake populations have wider and shorter head structure than river populations. River populations have more elongated and thin head structure that is typical river body formation for the river habitats with fast water current (McGuigan *et al* 2003).

İnsuyu River and Lake Gök populations of *P. crassus* and *P. egridiri* show considerably low genetic diversity and low heterozygosity. *P. egridiri* has critically endangered conservation status according to IUCN criteria. Both Lake Gök and İnsuyu river has been under heavily habitat destruction by water removing for agricultural activity that may leads to low genetics diversity and heterozygote deficiency.

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