4.5 Genetics sampling

4.5.1 Objectives of genetics sampling

The tools of population genetics provide methods for identifying a variety of attributes about a population. Of particular interest in fisheries are identification of aspects of the species (groups of species, species types (i.e. individual species), sub-species, stocks) as well as the structure, geographical range and boundaries of each of these species attributes.

Species identification can be useful for enforcement when mis-identification is common. For example, small yellowfin and bigeye tuna individuals can be confused.

For effective management of highly migratory tunas, it is important to identify appropriate stock boundaries (e.g. for Atlantic bluefin tuna). The approach is based upon the theory that population sub-division can result in the genetic differentiation of populations isolated by genetic drift and selection. To identify populations, the general approach is therefore to look at genetic diversity/dissimilarity at the population scale.

It is important to note that the absence of genetic evidence for population sub-structure does not mean that such sub-structure does not exist. Genetic differentiation between stocks sharing the same ocean basin may be fairly small, often of the same order of magnitude as the sampling error. Genetic studies of stock structure must therefore pay careful attention to experimental design and sampling protocols to maximise the signal-to-noise ratio in the data. However, genetic differentiation can be prevented where population sizes are large, and by migration. Theoretical models of nuclear gene product differentiation and mtDNA differentiation indicate that migration of the order of individuals per generation may be sufficient to prevent genetic differentiation. This rate of exchange is minimal in terms of the amount of exchange that would be needed to rebuild depleted populations on a time scale of interest to humans exploiting those populations. Given the limitations inherent in each individual method, the best approach to evaluating stock structure is a holistic one that draws on all the information available from genetic, demographic, ecological and life history studies (Waples, 1998).

4.5.2 Targeting samples

The specific targeting of samples to be taken will be defined by the aims of the sampling programme. Local sampling will define localised variations and mixing in populations through statistical analysis of genotypic data. Broader sampling will identify larger scale genetic differences and similarities. If the aim is to sample population natal characteristics, small individuals should be targeted, for example. These individuals should be within the natal area. In contrast, population scale examinations will require samples from across the known or suspected geographical range. Comparison of genetic profiles in different samples separated by space and/or time may provide evidence for multiple gene pools, or stocks.

4.5.3 Sample size

Available financial resources often limit sample size. Results of genetic analyses can be strongly influenced by sample size, however. The number of sites and years sampled will also increase the number of individuals to be sampled. In general, full programmes analyse over 100 samples per sampling unit (e.g. species, location, year, etc.). The actual required sample size will depend on the genetic differentiation between individuals of the species in different geographic areas (for example). Furthermore, studies have found that maximising sample sizes and temporal coverage is important. The results of studies based upon small sample sizes can produce false-positives (e.g. Ely *et al*., 2002). For all these reasons appropriate statistical advice should be sought at the programme planning phase to ensure that the defined sampling programme will enable the programme goals to be attained.

4.5.4 Sampling procedures

Cleanliness of samples

Contamination must be avoided. The knife must be cleaned (for example in ethanol) before cutting each fish. Contamination might occur if a cutting tool is used on different fish without cleaning. If contamination may have occurred, this must be noted on the form for that sample.

Sampling

Liver, heart and skeletal muscle tissues can be sampled, as well as blood, fin clips or a portion of the whole animal (generally larvae or juveniles). It is recommended that samples from the heart, liver and muscle be taken from an individual. Access to these tissues may be limited in particular fisheries, where the market value of the fish is condition dependent. White muscle may be the most appropriate tissue in this case. When sampling white muscle, remove a flap of skin approximately 9cm in diameter in the central body area between the dorsal fin base and lateral line, to expose the muscle.

Take around $4cm^3$ each (~5g) of muscle, heart and liver. Muscle and liver are recommended for DNA analyses. Clean, trim and wash in cold buffer if required (e.g. 50mM EDTA). Rinsing has been found in some studies to increase yields of closed circular mitochondrial DNA (mtDNA). Smaller pieces are recommended for DNA analysis, since this ensures the ethanol can penetrate the tissues for storage.

There are two different methods for storage of fish material: freezing and ethanol. The use of these alternatives is study dependent. Freezing is the best method for the storage of specimens for electrophoresis and other biological analysis (e.g. biochemistry and physiology), because of the liability of enzymes *in vitro*. Proper cryogenic storage will preserve enzyme activity and minimise breakdown (e.g. through the use of liquid nitrogen). Samples preserved in ethanol can ONLY be used for genetic studies, such as DNA amplification and sequencing. Samples can be stored in 96% ethanol.

Package the sample into plastic bags (when freezing samples) or into the vial of ethanol and then plastic bag (for ethanol) for storage. In each case, the bags should be labelled with (if available), date, vessel, suspected species, length, weight, sex, location of capture (latitude and longitude), tuna school type and school association, tissue type and a unique sample number. All samples from the same fish should be labelled with the same code. If this fish has been used in other studies (maturity etc.), the same code should be used throughout.

When freezing, samples should be frozen as soon as possible after collection. If this is not possible, the sample should be kept on ice until arrival at the laboratory. Samples should then be placed immediately in the freezer.

Shipping of frozen samples

Samples should be shipped to the required recipient on dry ice (within a stay-foam cooler is sufficient). If dry ice is not available, the cooler should be covered with ice and brought into the freezer for some days prior to shipping. Shipment should be carried out by Air Cargo System in regular flight as personal cargo. This approach is faster and cheaper than other delivery approaches. The “Air Waybill” document should be faxed to the recipient. This is the preferred approach. Alternatively, the invoice number for the shipment should be sent.

Shipping of samples in ethanol

Shipment of bags containing vials can be carried out by any regular mail system or other delivery system, to the recipient.

4.5.5 Analysis of samples

Analysis of tissue samples for DNA requires trained personnel and appropriate equipment. The general techniques will be described here, to ensure readers have an understanding of the roles of genetic analysis. For further details, see articles listed in the further reading section.

The tissue sample can be sub-sampled and small (e.g. 100mg, although this will depend on the sensitivity of the technique selected) samples can be incubated in 1ml of digestion buffer. The period of incubation and temperature will depend on the tissue and approach (see further reading). Alternative approaches for DNA extraction include standard phenol/chloroform procedures, followed by ethanol precipitation.

Species identification

Allozyme electrophoretic patterns and mitochondrial DNA (mtDNA) have proved useful for distinguishing different tuna species. DNA is the preferable target for examination, since DNA is the same in all cell types of an organism, while proteins can vary from tissue to tissue. DNA is also stable and provides more information for

analysis than protein. Furthermore, electrophoretic techniques have been shown to be less capable of species differentiation in Atlantic tunas (Bartlett and Davidson, 1991).

mtDNA haplotype analysis has also been used for species identification. For example, ATCO mtDNA haplotypes have been used to differentiate between bigeye and yellowfin catches. Ward (1995) noted that RFLP (restriction fragment length polymorphism) analysis of several mitochondrial genes permitted the unambiguous discrimination of all seven *Thunnus* species on the basis of exclusive haplotypes. This was supported by allozyme and mtDNA tests (Ward 1995).

Population discrimination

While allozymes have been analysed to quantify levels of genetic variation within and among populations, more powerful approaches are available. These include nuclear DNA microsatellite techniques, and analysis of the mtDNA control region (D-loop).

Although the value of mtDNA in identifying population subdivision has been well documented, analyses based solely on mtDNA haplotype frequencies might actually reflect sex-specific dispersal or migration patterns. This is because mtDNA is only passed down from the female of the parents. Furthermore, the non-recombining nature of the mtDNA genome causes it to behave as a single genetic locus, potentially reducing the power to detect significant genetic differentiation (Greig *et al*., 1999). Therefore, the most informative analyses of population structure combine multiple loci to test for similarity of phylogenetic patterns.

4.5.6 Analysis of results

Levels of genetic variation can be assessed in terms of the numbers of alleles per locus, and observed (H_{obs}) and Hardy-Weinberg expected heterozygosity (H_{exp}) (for nuclear DNA only). By comparing H_{obs} with H_{exp}, and testing for deviations from the Hardy-Weinberg equilibrium within samples, the significance of genetic variation can be assessed.

Homogeneity of microsatellite allele frequency can be assessed between temporal and spatial populations. Temporal samples from same spatial area can be pooled if they do not differ significantly in allele frequency. Spatial data can then be compared. Use of the sequential Bonferroni technique (Rich, 1989) is recommended to adjust significance levels for multiple simultaneous comparisons. Population differentiation can also be measured using analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992).

A further technique is the use of hierarchical analysis of nucleotide diversity as a measure of population differentiation (Holsinger and Mason-Gamer, 1996). This approach allows the examination of geographically structured populations using restricted site and DNA sequence data where the variation is not independently inherited. Populations are grouped based on the average time to coalescence for pairs of haplotypes. Results are depicted in a tree diagram that shows the relationship between populations after resampling the data 10,000 times. Significant P-values imply that the mean time to coalescence for two haplotypes drawn from the same node of a tree is less than that for two drawn from different nodes.

4.5.7 Further reading

APPLEYARD, S.A., P.M. Grewe, B.H. Innes, and R.D. Ward. 2001. Population structure of yellowfin tuna (*Thunnus albacares*) in the western Pacific Ocean, inferred from microsatellite loci. Marine Biology 139: 383-393.

BARTLETT, S.E. and W.S. Davidson. 1991. Identification of *Thunnus* tuna species by the polymerase chain reaction and direct sequence analysis of their mitochondrial cytochrome b genes. Can. J. Fish. Aquat. Sci. 48, 309-317.

ELY, B., D.S. Stoner, J.R. Alvarado Bremer, J.M. Dean, P. Addis, A. Cau, E.J. Thelen, W.J. Jones, D.E. Black, L. Smith, K. Scott, I. Naseri, and J.M. Quattro. 2002. Analysis of nuclear *ldh*A gene and mtDNA control region sequences of Atlantic northern bluefin tuna populations. Mar. Biotechnol. 4, 583-588.

EXCOFFIER, L., P.E. Smouse, and J.M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes-Application to human mitochondrial DNA restriction data. Genetics 131, 479-491.

GREIG, T.W., J.R. Alvarado Bremer, and B. Ely. 1999. Preliminary results from genetic analyses of nuclear markers in swordfish, *Xiphias gladius*, reveal concordance with mitochondrial DNA analyses. Col. Vol. Sci. Pap. ICCAT, 49(1): 476-482.

HOLSINGER, K.E. and R.J. Mason-Gamer. 1996. Hierarchical analysis of nucleotide diversity in geographically structured populations. Genetics 142, 629-639.

PUJOLAR, J.M., M.I. Roldán, and C. Pla. 2003. Genetic analysis of tuna populations, *Thunnus thynnus thynnus* and *T. alalunga*. Marine Biology 143, 613-621.

WAPLES, R. 1998. Identifying stock structure and resolving stock mixtures in tuna. Col. Vol. Sci. Pap. ICCAT 50, 207-208.

WARD, R.D. 1995. Population genetics of tunas. J. Fish Biol. 47, 259-280.