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Molecular Genetic Research on Terrestrial Plants and Animals in Micronesia over the Previous 60 Years[*] [1]

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Abstract— Early genetic studies in Micronesia focused on the use of protein electrophoresis to identify species and to measure genetic diversity within and among species. With the development of DNA sequencing techniques, many terrestrial species were discovered to be distinct from species on neighboring islands that share similar morphologies. With mitochondrial and nuclear DNA sequencing, and also DNA profiling (microsatellites), a number of endemic and introduced species were found to have lost genetic variation upon colonization and even to have undergone subsequent genetic bottlenecks. Other species were shown to have wide distributions maintained by considerable gene flow among islands. DNA sequencing and microsatellite studies have also been important in ongoing research to determine the geographic origin of agricultural pests and vertebrate invasive species. The ability to document the amount of genetic variation present in endangered species, keystone species, and invasive species offers important insights into the potential adaptability of these species to a changing environment. Considerable research opportunities exist for future population genetic studies, including the application of more advanced genetic methods, such as detection of natural polymorphisms, genome sequencing and gene expression studies. Such investigations could be of considerable value in conservation planning, environmental management, and sustainable resource use in Micronesia through the identification of the genetic basis of specific environmental adaptations.

Introduction

The present review will illustrate the development of genetic research on terrestrial plants and animals in Micronesia in the sixty years since Watson and Crick's publication of the structure of DNA. This time period also covers the years in which the University of Guam has grown from a teacher's college to a major research center for many of the islands of Micronesia. In concert with the progress of genetic research in the rest of the world, answers to genetic research questions in Micronesia have been highly dependent on the development of genetic technology, especially the polymerase chain reaction (PCR) and automated DNA sequencing as well as more recent genomic technologies.

The goal of this historical review will be to illustrate key progress in the types of population genetic questions that could be answered using relevant examples of the terrestrial flora and fauna of Micronesia. This review is therefore not intended to be comprehensive and naturally focuses on

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species that have received the most research attention because of their practical importance in agriculture and conservation management. Opportunities for future studies using new genomic methods, especially next generation DNA sequencing and transcriptome sequencing will also be briefly reviewed.

Perhaps the earliest genetic research on Micronesian animals and plants began with the use of species such as the fruit fly, *Drosophila*, to measure the frequency of induced mutations due to radiation from the nuclear testing in the Marshall Islands (Wilson & Stone 1958). The period before Watson and Crick's discovery of the structure of DNA also saw some limited attempts to diagnose species differences by looking at differences in chromosome number and morphology (karyotype) particularly for important cultivated plants (such as bananas and plaintains, *Musa* spp.) [Heslop-Harrison & Schwarzacher 2007] where cultivars were bred from clones that experienced spontaneous mutations.

Measurement of Protein Polymorphism Within and Among Populations

By the late 1960s, the technique of protein electrophoresis was able to quantify genetic variation in an array of enzyme loci that were common to many species. These genetic variants (alleles at a single enzyme locus, designated as allozymes) turned out to have a surprising amount of genetic variation (Mitton 1997). The maintenance of such genetic variation was subsequently explained by complex interactions between the forces of mutation, natural selection and genetic drift (Lewontin 1974).

One of the primary applications of allozyme studies to species in Micronesia was the use of this method to quantify the amount of genetic diversity remaining in several species of birds such as the Guam rail, *Rallus* (now *Gallirallus*) *owstoni* (Haig & Ballou 1995, Haig et al. 1994), and the Guam population of the Micronesian kingfisher (*Halcyon* (now *Todirhamphus*) *cinnamomina cinnamomina*)[Haig & Ballou 1995] after the population reduction caused by the introduction of the brown tree snake (*Boiga irregularis*) [Wiles et al. 2003, Rodda & Savidge 2007]. Generally, low genetic diversity was found relative to related species from continental areas. This loss of genetic diversity can result in decreased Darwinian fitness of such inbred populations compared to related outbred species (Crnokrak & Roff 1999).

However, it has often proved difficult to distinguish low genetic diversity due to initial colonization of Micronesian islands by a few founder individuals and a loss of genetic diversity due to the rapid population decrease due to predation by the brown tree snake. This ambiguity may now be resolvable with the use of museum specimens collected prior to the population explosion of the brown tree snake combined with current methods of measuring intra-population genetic diversity (see below).

In principal, it should be possible to determine whether two populations represent two different biological species using allozymes if each population is fixed for different alleles at one or more loci. Such a question has practical importance in reintroduction programs such as supplementing the Guam population of the Mariana crow (*Corvus kubaryi*) with birds from the more numerous Rota population.

Allozymes have also been successfully utilized to determine the hybrid origin of a parthenogenetic species of gecko found in Micronesia, *Lepidodactylus lugubris* (Radtkey et al. 1996). In addition, allozyme data have also been used to test whether the colonization of cycads in the islands of the South-West Pacific resulted from dispersal or vicariance (Keppel et al. 2008).

At the present time, however, information derived from DNA sequencing and more modern methods of measuring genetic polymorphism (such as DNA profiling using microsatellites) have now largely superseded the use of allozymes in assessing genetic variation.

DNA Fingerprinting

One method developed in the mid-1980s by Alec Jeffreys, minisatellite DNA fingerprinting, revealed more genetic diversity than did allozymes by using labeled probes to create the familiar "bar-coded" fingerprint (Gitschier 2009). This method used repetitive DNA sequences and a labeled probe to develop a set of bands that were inherited either from the father or the mother. Its principle statistic, percentage of band sharing among individuals, was employed as one method to determine that the Rota population of the Mariana crow was more inbred than the few remaining Mariana crows on Guam (Tarr and Fleischer, 1998). Given that the Saipan population was much larger at the time, this led to the further conclusion that the Saipan population had been founded by a few birds colonizing from the Guam population. A second practical application compared the degree of inbreeding as calculated from pedigrees (via "mean kinship") and with that calculated using the percentage of band-sharing in DNA fingerprints in the 16 founder Guam rails (Haig et al. 1994).

A similar method, Southern blotting, used a labeled probe and genomic DNA to identify the presence of specific genes or DNA sequences in cultivars such as those involved in nitrogen fixation in several legumes (You et al. 2002). This method of gene screening was later largely replaced by direct amplification of the DNA segment of interest followed by automated DNA sequencing.

The Polymerase Chain Reaction (PCR)

The invention of the automated polymerase chain reaction (PCR) by Kary Mullis in 1986 greatly expanded the types of samples that could be genotyped. By amplifying small amounts even of degraded DNA, PCR allowed samples from museum specimens or non-invasive sampling (from hair, skin, feathers, and bone) to be amplified and genotyped for comparison with genotypes of individuals from extant populations (Piggott & Taylor, 2003). This method permitted individual gene loci to be targeted using primers designed for that locus in species with more well-studied genomes.

For species where little or nothing was known of the genomes, such as the ironwood tree (*Casuarina equisetifolia*), anonymous nuclear loci are targeted with PCR using randomly amplified polymorphic DNA (RAPDs) to distinctly fingerprint different populations (Ho et al. 2002). This study revealed significant genomic diversity among trees from different islands that might be used to increase the genetic resistance to the diseases that are killing ironwood trees here on Guam. A more advanced and reproducible form of DNA fingerprinting, amplified fragment length polymorphism (AFLPs), was successfully used to determine the contributions of wild species of breadfruit (*Artocarpus*) to the genomes of important cultivated varieties, a pattern that turned out to be closely related to the known dispersal of human populations in Oceania (Zerega et al. 2004).

PCR also greatly facilitated DNA sequencing by providing significant amounts of DNA, for automated sequencing via the Sanger method. Mitochondrial DNA was the most favored type of DNA for PCR as it is found in numerous copies in every eukaryotic cell and was therefore available even in degraded samples that might be collected via non-invasive sampling of shed hairs, skin, or feathers. Mitochondrial DNA has been used to detect the number of maternal lineages in endangered species such as the Mariana crow and the Okinawa rail (*Gallirallus okinawae*) [Ozaki et al. 2010], which is a congener of the Guam rail. Future mitochondrial sequencing of the Guam rail could allow determination of the number of maternal lineages in this endangered species in comparison with a closely-related and widely-distributed species (*Gallirallus philippensis*).

Mitochondrial DNA phylogenies have been used to detect convergent evolution among the Pacific reed warblers. Populations once found in different Mariana Islands all once placed in the Guam species (*Acrocephalus luscinia*) have now been shown to be due to independent colonizations (Cibois et al 2011). This result makes any possible "reintroduction" of the

nightingale reed warbler to Guam now dependent on using a related species, which is more problematic from both a regulatory and an ecological perspective. Unfortunately, the new genetic analyses have shown that some extirpated Micronesian populations are now actually extinct endemic species. In contrast, future DNA sequencing of mitochondrial DNA may possibly show that populations of birds that were considered island endemics are actually only sub-species of related birds still surviving on other Micronesian islands (Baker 1951).

The role of natural selection for flightlessness in island rails and crakes has been strongly implicated by Kirschmann's (2012) and Slikas' et al. (2002) mitochondrial DNA studies that showed multiple lineages independently losing the ability to fly. Mitochondrial DNA sequencing has also shown monophyly of the swiftlets in the genus *Aerodramus* (including *Aerodramus vanikorensis bartschi*, the endangered Mariana gray swiftlet) that use a significant behavioral innovation (echolocation) [Lee et al. 1996].

Both nuclear gene loci and mitochondrial loci are now being amplified via PCR with individual nuclear, unlinked loci favored as providing independent tests of species and gene trees. The land snail radiations in Micronesia, including the famous partulid snail radiation in Micronesia and elsewhere in Oceania, have benefited from such a combined approach to molecular phylogenies (Goodacre & Wade, 2001, Rundell 2008). Patterns of colonization and speciation elucidated with molecular genetics methods have identified endemic species that can be prioritized for conservation, including captive breeding.

PCR was also used to determine the source populations for the introduced skink (*Carlia* fusca). The most surprising conclusion from this study was that the main food for the brown tree snake at the present time (*C. fusca*) was perhaps introduced at the same time as the snake itself (Austin et al. 2011). Past genetic studies and ongoing research are also using both mitochondrial and nuclear DNA to determine the geographic origin of the brown tree snake or snakes that colonized Guam just after WWII (Rodda & Savidge 2007, Richmond et al. 2012). The single mitochondrial DNA genotype and bi-allelic polymorphism for nuclear genes indicate that the Guam population of the snake was most likely founded by only a few individuals inhabiting a restricted area in the source region (the Admiralty Islands).

DNA Barcoding and the "Barcode of Life" Project

In the 1990s, several biologists at the University of Guelph in Canada decided to genotype each biological species on earth using a set of mitochondrial and, for plants, some chloroplast and nuclear gene loci (Hollingsworth et al. 2011) in a global project named the "Barcode of Life" (Savolainen et al. 2005). This method uses mitochondrial DNA to uniquely identify animal species. It has been used in Micronesia to identify specific aphid genotypes that attack native and cultivated plants and that often spread serious viral diseases (Fottit et al. 2008). However, other genes must also be included in studies of insect host race evolution, since such changes may or may not involves changes in DNA bar-coding loci of insects and their host plants (Reddy et al. 2005, Rubinoff et al. 2010).

Plant loci now selected for the "Barcode of Life" project have been used to place many native tree species, a number of which have medicinal value, within their respective plant families. DNA barcoding has also been used to differentiate between plant or animal species that are superficially similar, but that actually represent distinct biological species or evolutionary lineages. This is important since different Micronesian species of medicinal plants in the same genus as those used in Indian or Chinese herbal medicine, e.g. *Aglaia* (Muellner et al. 2005, Kinghorn et al. 2011), *Tinospora* (Ortiz et al. 2007) and *Morinda* (Razafimandimbison et al. 2009) may have distinct secondary compounds that can alter their medicinal effects.

PCR-Based DNA Fingerprinting using Microsatellite Loci

The polymerase chain reaction led to replacement of both allozymes and minisatellite DNA fingerprinting with amplification of simple sequence repeats (such as GC_n) designated as microsatellite loci. Different numbers of repeats of such a sequence give many more alleles per locus as compared to allozymes. Microsatellite genotyping (known as "DNA profiling" when applied to humans) has the advantage over minisatellite fingerprinting because all the extensive population genetic theory developed for protein (allozyme) loci is applicable to microsatellite loci.

Microsatellite genotyping has greater statistical power to detect a population bottleneck that results in a loss of genetic variation as compared with allozymes. PCR allows DNA from museum specimens and from non-invasive sampling (Pigott & Taylor 2006) to be genotyped with microsatellites (Selkoe & Toonen 2006). Therefore, historical and extant populations can be compared to determine how much genetic diversity has been lost. Such studies with the endangered greater prairie chicken, for example, have shown that many more alleles were present at certain microsatellite loci in past populations (Bellinger et al. 2003). Such loss of genetic diversity is considered an indicator of a possible loss of future adaptability of these populations to changing environments. If different alleles have been lost in distinct subpopulations, managed gene flow to increase allelic diversity can sometimes be a means of reducing inbreeding depression (Hedrick & Kalinowski 2000).

The use of mitochondrial DNA sequencing and microsatellite genotyping can also reveal that populations once thought genetically distinct, such as the Guam and Rota populations of the Mariana fruit bat (*Pteropus mariannus*) are actually members of a single species with considerable gene flow among all the Mariana Islands (Brown et al. 2011). This result caused the status of the Guam population to be reduced from "endangered" to "threatened" (along with the fruit bat populations in the Commonwealth of the Northern Marianas). This study also showed that no significant gene flow was present between the Marianas and Palauan fruit bats, which therefore represent good biological species .

Whole Genome Approaches and Future Developments

A fundamental problem in evolutionary studies has always been detection of genes that are under natural selection, especially when recent population bottlenecks and selective sweeps can leave similar genetic signatures (Stephan 2011). Often, this problem is exacerbated by an absence of knowledge about the structural proteins, enzymes or regulatory sequences involved in a specific adaptation. Despite heroic and long-standing efforts, few allozyme loci have been successfully associated with fitness differences, whereas studies of the MHC loci in vertebrates have been convincingly associated with fitness differences (Radtkey et al 1996, Bernatchez & Landry 2003).

With the ability to sequence entire genomes, it has become possible to scan genomes for specific polymorphisms (called Single Nucleotide Polymorphisms or SNPs) and look for linkage to specific phenotypes. Such Genome Wide Association (or GWA) studies have been applied mainly in medical genetics, but they can be applied to any phenotype, especially those determined by more than one or two gene loci. This method can be combined with automated sequencing of DNA regions surrounding SNPs to determine what proteins or regulatory DNA may be involved in a particular phenotype (Helyar et al. 2011). Once such proteins or regulatory DNA have been identified, they can give clues to the biochemical mechanism producing that adaptation. One suggested application (Kirchmann, 2012) has been to identify loci involved in the parallel evolution of shortened wings and reduced flight muscles in the closely-related flightless rails of the Pacific.

A second method to scan genomes for loci involved in adaptations to the environment is the use of Expressed Sequence Tags (ESTs) to identify DNA sequences that correspond to transcribed genes as population markers. This method has been used to show microgeographic spatial variation

in the endangered cycad (*Cycas micronesica*) on Guam, perhaps based on soil characteristics. Furthermore, this method has been used to identify selective sweeps that rapidly increase the frequency of a new allele of high fitness. Different EST variants were identified as candidates for balancing or directional forms of natural selection (Cibrian-Jaramillo et al., 2010).

DNA sequencing has now produced highly sophisticated next generation (NexGen) DNA Sequencers that can reconstruct whole genomes for comparison within and among species (Shendure & Ji 2008). These new DNA sequencers are also recommended for cost-effective isolation of microsatellite loci in nonmodel species (Guichoux et al. 2011), including endangered species (Jennings et al. 2011). An approach to producing a great deal of information on coding sequences for use in population genetics and molecular ecology is next generation sequencing of transcriptomes (Gayral et al., 2011). All such next generation sequencing methods are likely to become much more cost effective as the technology and associate software continue to evolve in a highly competitive environment (Glenn, 2011).

Indeed, some scientists are now hypothesizing that "de-extinction" (the reconstruction of the entire genome of extinct organisms in functioning individuals) may be technically possible in the near future (Sherkow & Greely 2013). These researchers envision using an extant relative, such as the band-tailed pigeon (*Patagioenas fasciata*), to give a genome sketch that could be modified by sequencing the extinct passenger pigeon (*Ectopistes migratorius*) DNA sequences to reconstruct an extinct species. At present, however, population genetics and genomics are much more likely to be used to justify the reintroduction of extinct or extirpated species by demonstrating that related island species or populations are close enough genetically that they may be considered the ecological equivalents of the species that has disappeared locally (Haig 1998). Population genetics has become an essential component of ecosystem reconstruction though its role in justifying reintroductions (Tarr & Fleischer 1999) or translocations may always arouse controversy. Genetic differences are not easily translated into the reproductive and ecological barriers that separate biological species in nature. Therefore, the use of considerably more genetic information than is provided by such biodiversity indices as DNA barcodes is always to be recommended in making practical decisions on reintroductions or translocations in conservation biology (Baker et al. 2009).

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