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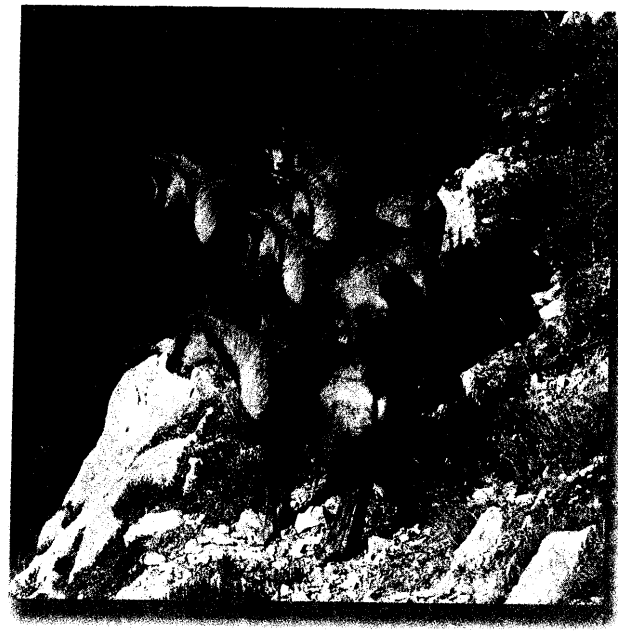

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GUIDELINES ON THE USE OF MOLECULAR GENETICS IN REINTRODUCTION EFFORTS

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ABSTRACT

The use of molecular genetics can play a key role in reintroduction efforts. Prior to the introduction of any individuals, molecular genetics can be used to identify the most appropriate source population for the reintroduction, ensure that no relic populations exist in the reintroduction area, and guide captive breeding programs. The use of molecular genetics post-reintroduction has received less attention. Yet, it is after the reintroduction that molecular genetics combined with non-invasive genetic sampling provide the greatest results. Most reintroduction programs install a weak monitoring program at best after the release of animals. With the advent of molecular techniques it is now possible to track individuals and populations through time by sampling their feces or hair without ever handling or disturbing the animals. This manuscript reviews the use of molecular genetics both pre- and post- reintroduction of wildlife.

INTRODUCTION

The field of molecular genetics can provide powerful tools to aide in the reintroduction and augmentation of wildlife populations. In this document I attempt to provide useful information for those interested in applying molecular genetics to reintroduction efforts, describe the limitations and pitfalls of these tools, and when applicable, describe what is being done to increase the utility of molecular genetics in reintroduction programs.

In general there are two distinct phases where molecular genetics can aide in reintroduction efforts. The first phase is prior to any animal being released into the wild. In this phase DNA-based methods can be used 1) to understand the population structuring of the target species so the proper source population can be selected, 2) to sample areas where the native population is thought to be extinct to ensure no native population still persists in low numbers (that is, to confirm one is really doing a reintroduction and not an augmentation), and 3) to assist in captive breeding efforts.

The second phase where molecular genetics can help reintroduc-

tions is post-release of individuals. In this phase DNA-based approaches can: 1) help track individuals through time, 2) help assess overall size of the reintroduced population post release, and 3) be used to monitor the basic "genetic health" of the population. Below I detail each of these uses of DNA-based approaches.

However, before detailing the use of DNA-based approaches two caveats must be stated. First, this manuscript only provides general guidelines. One would need to know the species, location, and exact situation of the reintroduction before specific recommendations could be made. Second, while both population genetics and molecular genetics (DNA-based approaches) are incredibly useful for reintroductions, they should not come at the expense of demographic, habitat, sociological, and other ecological approaches. The *combination* of understanding a species' habitat needs, ecological requirements, behavior, population dynamics and genetics is essential for having a successful reintroduction.

Molecular Genetics versus Population Genetics

As a point of clarification, using molecular genetics is different than applying population genetics to the problems of reintroducing wildlife populations. Molecular genetics, as typically applied to wildlife, is the use of multiple segments of a species' DNA code to make inferences about the individual, the population, or relationship of individuals, populations, and species to larger taxonomic units; population genetics, on the other hand, is the study of the distribution of and change in gene frequencies under the influence of evolutionary forces. Population genetics can be conducted without examining DNA or any molecular structure, whereas molecular genetics requires the examination of DNA, RNA or other molecular structures. Population genetic theory has much to add to the field of reintroduction of wildlife populations and at times molecular genetic data is used in a population genetic framework to aide in reintroductions. Population genetics is especially pertinent to reintroduction efforts since most reintroduction efforts involve placing small populations back into the wild, and the field of small population genetics is well developed (Hedrick, 2000; Hedrick, 1995; Hartl & Clark, 1989). However, there are many instances where the use of genetic data can be used without applying population genetics theory; therein lies the difference. In this manuscript I focus on what information molecular genetics can add to reintroduction efforts.

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Apennine chamois *Rupicapra pyrenaica ornata*. Gordon Luikart and Kristy Pilgrim provided helpful comments on earlier drafts of this manuscript.

USE OF MOLECULAR GENETICS PRE-INTRODUCTION

"Reintroduction and conservation projects aimed at reducing the chance of extinction of native stocks, could almost certainly be improved by carrying out preliminary genetic analyses"

Vernesi et al. (2003)

Species Structure / Choosing Source Population

Geography, history, life history, ecology, behavior, and population dynamics influence how a species becomes structured across a landscape. A geographic barrier for one species is inconsequential for another. Thus, a vagile species may act as one population across a mountain chain, whereas a more sessile species may divide into multiple populations, or even subspecies, across the same landscape. For instance, in the Western United States, big horn sheep (*Ovis canadensis*) are highly structured with herds on different mountains chains splitting into distinct populations (Forbes & Hogg, 1999), whereas lynx are completely unstructured across their entire North American distribution (Schwartz et al., 2002; Rueness et al., 2003; Figure 1).

The goal of reintroduction efforts should be to re-introduce individuals as closely related genetically, behaviorally, and morphologically to the population that historically persisted in the reintroduction area. Knowing how a species structures across a landscape and what local adaptations it possesses become essential information for reintroduction efforts. While the specific local adaptations of a species to a location are often unknown, it is known that moving individuals from a population that is not adapted to a new location can lead to failed reintroduction efforts (Tallmon et al., 2004). Molecular genetic methods can provide insight into when local adaptations may exist, thus guiding reintroductions.

The first step in a reintroduction effort should be to understand the phylogenetic and population substructure of the species to be reintroduced. A common approach is to define evolutionary significant units (ESUs; Waples, 1991; Crandall et al., 2000; Moritz et al., 1994), populations, or other phylogenetic units that structure the species. While the specifics criteria for defining ESUs are hotly debated, the general principle is to find a population unit that merits separate management due to its reproductive isolation from other populations and its unique adaptations (Waples, 1991; Crandall et al., 2000). For instance, North American brown bears (*Ursus arctos*) have four distinct ESUs based on mitochondrial DNA data (Waits et al., 1998). This information is essential if one were to conduct North American brown bear reintroductions, as the best source population is likely

one that is within the same ESU.

By definition, a reintroduction is replacing the animals that were once in a location with individuals from another location. Therefore, the genetic composition, physiology, behavior and morphology of the extinct population may not be known (unless individuals had been removed from the reintroduction area and placed into a captive breeding program). When little is known about the extinct population, surrogate data must be brought to bear; these data may be in the form of historical accounts of the extinct population, former taxonomic descriptions of the extinct population, or occasionally prior molecular genetics work conducted prior to the population extinction event. Alternatively, as with many reintroductions, it can be assumed that the geographically closest population is physiologically, genetically, and morphologically similar to the extinct population.

In the past decade the field of "ancient DNA" has flourished (Cooper et al., 1996; Leonard et al., 2000); that is, researchers can now extract DNA from pre-fossilized bones. If museum specimens are available from an extinct population that is being replaced through a reintroduction effort, it is now possible to compare the extinct population's DNA to the putative source population (Leonard et al., 2000; Schwartz et al., Unpublished data on wolverine, *Gulo gulo*). In other words, with ancient DNA it is now possible to use molecular genetic information to choose the source population that is genetically closest to the extinct population. Using the measure of "genetically closest" is more defensible compared to using the arbitrary decision of using the "geographically closest" population for reintroduction source material. Reintroduction efforts of the Mexican gray wolf (*Canis lupus baileyi*) demonstrates how ancient DNA work can guide management. Leonard et al. (2005) examined 34 DNA sequences from pre-extermination wolves and found that the Mexican wolf clade contained DNA sequences typical of the Northern clade. Thus, the introduction of individuals from the northern clade into the Mexican gray wolf captive breeding population may help enhance the adaptive potential of reintroduced stocks (Leonard et al., 2005).

While using scientific data for choosing a source population for reintroductions is preferable, it is not without risk. Most genetic data used to identify a source population is based on neutral genetic markers. This data does not tell us anything directly about the traits that may be under selection in different parts of a species range. Furthermore, it is possible that the ESU analysis may show little structuring, but in fact, there are populations that have adaptive traits. For instance, populations on the periphery may be under strong selection for some traits, but because of high gene flow from a core population the peripheral populations may look the same as the core population. New genetic techniques, called single nucleotide polymorphisms (SNPs), will lead to new insights into traits that are under

selection, aiding in the identification of ESUs. For instance, willow warbler subspecies (*Phylloscopus trochilus*) in Scandinavia are not differentiated in their mitochondrial or nuclear DNA, despite differences in migratory behaviors (Bensch et al., 2002). However, SNP research has identified a region of the genome under selection that differs between migratory types (Bensch et al., 2002). Using these new types of markers to better identify adaptive differences will greatly improve the efficacy of reintroductions.

Using DNA data (even SNP data) for choosing a source population may be considered an arrogant approach. That is, we think we can learn enough about the species via genetic methods to identify the best populations for reintroduction, despite admitting that we know nothing about most traits under selection. Thus, some reintroduction programs have opted for a "mix approach" where animals from multiple populations are introduced with the notion that natural selection will sort out the most appropriate phenotypes over time (Tordoff & Redig, 2001; Temple & Cade 1988).

Finally, science is a learning process; today's best information may be obsolete tomorrow. For instance, Waits et al. (1998) suggest four ESUs for brown bears in North America. However, more recent genetic data using permafrost-preserved brown bear specimens shows that approximately 36,000 years ago the brown bear had higher and more uniform genetic diversity across the range. Thus, given that ESUs are purportedly long-term divisions of a taxa, and given that the brown bear structure today is likely only approximately 15,000 years old, Leonard et al. (2000) suggest brown bears should be managed as one ESU. Overall, it needs to be understood that the use of all data, genetic or otherwise, for the guidance of reintroductions is only as good as the current state of knowledge. As science advances, so will our understandings as to the best source population for reintroductions.

Using DNA-Based Approaches to Survey for Extant Populations

As previously discussed, when one undertakes a reintroduction effort it is already assumed that the local population is extinct. Many times this assumption is correct, yet other times it is not. In difficult to access areas, with low human population densities, refugia populations often persist. Prior to any reintroduction effort detailed surveys for the presumed extinct species should be conducted. If the species is found (and in fact not extirpated), other options become available such as the initiation of captive breeding programs or habitat improvement projects. In fact, there may be a higher conservation value of a re-discovered species, or the land which it occupies, compared with a reintroduced population.

Vinkey et al (In Press) have shown that many fisher (*Martes pennanti*) reintroductions in the Rocky Mountains of Montana and Idaho were

not reintroductions, but were actually augmentations. These augmentations used individuals from nearby population that were genetically distinct from the native fisher (Vinkey et al. In Press). The consequences of these augmentations have lead to a genetically mixed population (native and reintroduced) of Rocky Mountain fisher. Had prior surveys been conducted it would have been possible to preserve a "pure" Rocky Mountain fisher population. Likely, if the native refugia was discovered prior to reintroduction, scarce conservation funding would have been used to improve habitat and aide the existing population, instead of funding reintroduction efforts with individuals of a different stock. Furthermore, there is some anecdotal information that the reintroduced individuals from non-native stocks have lower fitness in the Rocky Mountains than the native fisher.

Interestingly, the success rate for re-introductions with a wild source population is higher (31%) than for re-introductions that use captive populations (13%; Fischer and Lindemayer 2000). While many factors contribute to this result, it would not be surprising if part of the reason that wild sources are more successful is that these introductions don't use mixed stocks often found in captivity.

Non-invasive genetic surveys are a powerful way which we can survey difficult to access terrain to determine if refugia populations exist. Non-invasive genetic sampling is the collection of samples (usually hair, feces, or urine) from a species without having to handle or immobilize the individual. This can be accomplished by inducing the animal to leave a non-invasive sample at a specific location, by back-tracking an animal to obtain a non-invasive sample, by moving along transects to find samples, or by using conservation detection dogs to locate samples (www.workingdogsforconservation.org; McKelvey et al., In Press; Smith et al, 2003; Zielinksi et al., In Review). Once the non-invasive sample is collected, DNA can be extracted, and the individual or species that yielded the sample can be identified. Non-invasive genetic surveys are cheap to deploy and provide the ability to definitively identify the species that left the sample (Mills et al., 2000). All reintroduction efforts should undertake non-invasive surveys of putatively extinct populations prior to the movement of wildlife.

Captive Breeding

Reintroductions sometimes rely on captive breeding programs to bolster the numbers of an endangered species before reintroduction into the wild. Some of these captive breeding programs use molecular genetics to help select which individuals to breed. Ultimately, the goal of captive breeding programs is to retain high levels of genetic diversity for long periods of time. Throughout the past two decades there have been many suggestions as how best to accom-

plish this goal (Haig et al., 1990; Earnhard, 1999). The most common techniques used to maximize levels of genetic diversity are to 1) maximize the effective population size, 2) maximize the avoidance of inbreeding and minimizie outbreeding, and 3) minimizing kinship within the captive bred population (Frankham et al., 2002). At times there have been other suggestions for using molecular genetics in captive breeding programs, such as selecting for particular genes usually associated with disease resistance, however, these approaches have met with much criticism (Haig et al, 1990; Vrijenhoek et al., 1991; Miller et al., 1991, but see Hughes, 1991; Aguilar et al., 2004 for an opposing view). In the remainder of this section I discuss how molecular genetics can contribute to the three common approaches used for maximizing levels of genetic diversity in captive breeding programs.

Maximizing the effective population of the captive population can be accomplished by several means; most commonly this is performed by equalizing the family sizes, equalizing the sex-ratio of the breeding population, maintaining stable population sizes, or delaying the breeding of individuals to maximize the generation length (For a full review see Frankham et al., 2002). While population genetic principles are the basis for each of these methods, molecular genetic can play a substantial role. Specifically, molecular genetics is best applied when the paternity of an individual is in doubt. For many species, breeding outside the pair (extra-pair mating) and having multiple paternity broods or litters is common (Griffith et al., 2002). When paternity is in question, molecular genetic approaches can be applied to identify the parents of offspring. The most common molecular tool for identifying paternity is the use of microsatellite DNA markers, which are bi-parentally inherited (Marshall et al., 1998). For example, it was discovered in a captive breeding colony at the Dublin Zoo that 9.6% of the breeding of Chilean flamingos (*Phoenicopterus chilensis*) occur outside the bonded pair (Farrell et al., 2000). The use of microsatellites can determine the true identity of the fathers. Accurate knowledge of parentage will aide curators in equalizing family sizes when planning future pairings or for evaluating which individuals should be re-released into the wild. This will be most beneficial in captive situations where multiple breeding-age individuals are housed together.

Most captive breeding populations will suffer inbreeding depression given the small number of individuals maintained in captivity. Frankham et al. (2002) reviewed captive breeding programs and noted that even with pooling between captive facilities, the mean effective population size of a species in captivity is only 41 individuals. Management guidelines for avoiding inbreeding depression in captivity are the same as that for maximizing the effective population. Where molecular genetics may be more pertinent is in minimiz-

ing outbreeding. Outbreeding depression is the reduced fitness of offspring from matings between genetically divergent individuals due to either mixing of locally adapted traits or the disruption of co-adapted gene complexes (Templeton, 1986; Tallmon et al., 2004). Molecular genetics can be used to resolve taxonomic uncertainties (Frankham et al., 2002) and identify proper units for conservation (Moritz, 1994; Waples, 1991; Crandall et al., 2000; see above). These units often take into account both the reproductive isolation (distinctiveness) of populations and the adaptive differences between populations. Once these distinct units are identified, usually by using molecular genetic data, as well as morphological, behavioral, and historical information, it is recommended that individuals from individual conservation groups be managed separately. For example, orangutans (*Pongo pygmaeus*) were initially managed in captivity as one species; however, molecular genetic evidence suggests that the Sumatran and Borneo populations are two distinct groups with possible local adaptations (Warren et al., 2001). Thus, captive breeding programs now prevent interbreeding between Sumatran and Borneo individuals, especially for those individuals that may be re-released into the wild.

Minimizing kinship has been recommended as the best way to maintain genetic variation in a captive breeding population (Frankham et al., 2002). This approach uses pedigrees to choose breeding pairs that share the fewest relationships in the population. Thus, the contribution of founders is kept equal over time. While this approach uses genetic information (i.e., the pedigree), it does not specifically use molecular genetic information. Molecular genetics can assist by resolving uncertainties in the pedigree through parentage analysis. This can be accomplished when the individuals are no longer alive, but genetic samples such as blood or tissue has been archived. For example, Jones et al. (2002) were able to refine the whooping crane (*Grus americana*) studbook by incorporating microsatellite DNA analyses. Most importantly, they were able to resolve unknown genetic relationships between the founder individuals, which without molecular genetic information, are assumed to be unrelated.

Overall, while captive breeding programs rely heavily on population genetics theory, there are often opportunities for molecular genetics to assist in clarifying unresolved relationships between individuals. This is mostly accomplished through the use of parentage analysis to help identify extra-pair matings, mixed broods, and ambiguities in the pedigrees.