Review

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Which genetic marker for which conservation genetics issue?

Conservation genetics focuses on the effects of contemporary genetic structuring on long-term survival of a species. It helps wildlife managers protect biodiversity by identifying a series of conservation units, which include species, evolutionarily significant units (ESUs), management units (MUs), action units (AUs), and family nets (FNs). Although mitochondrial DNA (mtDNA) evolves 5–10 times faster than single-copy nuclear DNA (scnDNA), it records few traces of contemporary events. Thus, mtDNA can be used to resolve taxonomic uncertainties and ESUs. Variable number of tandem repeats (VNTRs) evolve 100–1000 times faster than scnDNA and provide a powerful tool for analyzing recent and contemporary events. VNTR analysis techniques include polymerase chain reaction (PCR)-based microsatellite assays and oligonucleotide probing. Size homoplasy problems in PCR-based microsatellite assays can strongly affect the inference of recent population history. The high homozygosity in endangered species is reflected in a relatively low number and level of variability in microsatellite loci. This combined with "allelic dropout" and "misprinting" errors contributes to the generation of highly biased genetic data following analyses of natural populations. Thus, in conservation genetics, microsatellites are of limited use for identifying ESUs, MUs, and AUs. In contrast to PCR-based microsatellite analysis, oligonucleotide probing avoids errors resulting from PCR amplification. It is particularly suitable for inferring recent population history and contemporary gene flow between fragmented subpopulations. Oligonucleotide fingerprinting generates individual-specific DNA banding patterns and thus provides a highly precise tool for monitoring demography of natural populations. Hence, DNA fingerprinting is powerful for distinguishing ESUs, MUs, AUs, and FNs. The use of oligonucleotide fingerprinting and fecal DNA is opening new areas for conservation genetics.

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Abbreviations: AU, action unit; **CR**, control region; **ESU**, evolutionarily significant unit; **FN**, family net; **mtDNA**, mitochondrial DNA; **MU**, management unit; **scnDNA**, single-copy nuclear DNA

1 Introduction

The biological diversity of our planet is rapidly diminishing as a direct and indirect consequence of human behavior. A large number of species are already extinct, and the populations of many others have been reduced to levels where they risk extinction. Species are being lost at a rate that far exceeds the emergence of new species. The current extinction problem has been called the 'sixth extinction', as its magnitude compares with that of the other five mass extinctions revealed in geological records [1]. In the past two decades, genetic technologies have been used to determine in precise detail the prospective status of several endangered species. Such approaches have provided important insights that have critically affected management decisions and produced tangible benefits for the species studied. With the application of molecular techniques in genetic studies of endangered species, conservation genetics has become a distinct discipline. The

science of conservation genetics is a mixture of ecology, molecular biology, population genetics, mathematical modeling, and evolutionary systematics (the construction of family relationships). Conservation genetics is the use of genetics to preserve species as dynamic entities capable of coping with environmental change. Conservation genetics encompasses genetic management of small populations, resolution of taxonomic uncertainties, defining management units within species, and the use of molecular genetic analyses in forensics and understanding species biology [1].

Although population genetics is a principal component of conservation genetics, it has different concerns. Population genetics chiefly focuses on the processes and mechanisms by which evolutionary changes are made. It provides the genetic underpinning for all evolutionary biology. The goal of population genetics is to understand the effects of various forces that result in evolutionary changes in species over time [2]. In contrast to population genetics, conservation genetics deals with the effects of loss of genetic diversity and recent changes in genetic structuring on the long-term survival of endangered species. Because endangered species have small and/or declining populations, and small populations suffer from inbreeding and loss of genetic diversity resulting in elevated extinction risks, a major concern in conservation genetics is the effect of small population size.

The technical advance of molecular markers has led to the blossoming of genetic analysis of populations in the last decade; however, the indiscriminate application of genetic markers used for population genetics to conservation genetics can potentially lead to inappropriate interpretations. This is due to the two fields having differences in content (historic/contemporary genetic variation), population size (big/small populations), time window (over past/recent events), and purpose (understanding evolutionary forces/exploring effects of eroded diversity on survival). Population genetics emphasizes the roles that different evolutionary forces play over time on the current population structure, which can be deduced from molecular markers of fast and/or slow evolutionary rates. In contrast, conservation genetics highlights the effects of contemporary genetic structuring on preserving endangered species as dynamic entities. This requires sensitive molecular markers in order to glean abundant and appropriate data from small populations.

An ISI web of knowledge search revealed that the expressions ‘population genetics’ and ‘conservation genetics’ appeared in 2837 and 372 articles between 1997 and 2003, respectively. This comparison shows that the discipline of conservation genetics is still in its infancy relative to population genetics. Undoubtedly, many conservation geneticists will apply the genetic markers used in population genetics to conservation genetics. In this review, we intend to show that the two popular marker classes in population genetics, namely mitochondrial DNA (mtDNA) and microsatellites, are of limited use in conservation genetics. Instead, oligonucleotide fingerprinting is more appropriate because of its high-resolution power in small populations.

2 Mitochondrial DNA markers

It is universally argued that mitochondria originate from bacteria. During organelle evolution the original bacterial genome has become progressively smaller as most of its genetic material has been transferred to the nucleus and other organelles [3, 4]. Shrinkage of mtDNA during evolution has resulted in mtDNA, which is linear in plants, becoming a closed circular DNA molecule in animals. Thus, plant mtDNA has distinct evolutionary dynamics compared with animal mtDNA, including high rates of rearrangement, duplication and incorporation of foreign DNA, and an evolutionary rate 50–100 times lower than for vertebrate mtDNA [4]. Accompanying the shrinkage of mtDNA is an increasing number of mtDNA genes being transposed to the nucleus and forming a superfamily of nuclear mitochondrial DNA (*Numt*), which are identified as pseudogenes because of differences between nucleus and cytoplasm genetic codes. The mitochondrial-like sequences can exist in high copy number with little difference in sequence among members in many organisms, making mtDNA of little practical value for population genetics studies in these groups [5]. These problems associated with plant mtDNA and unwanted *Numt* genes in animals were previously discussed by Zhang and Hewitt [5].

The mitochondrial genome comprises a circular ‘chromosome’ of DNA. Animal mtDNA ordinarily contain 36 or 37 genes; two for ribosomal RNAs, 22 for tRNAs and 12 or 13 for subunits of multimeric proteins of the inner mitochondrial membrane. In addition, there is a noncoding sequence termed the control region (CR) due to its role in replication and transcription of mtDNA molecules. Exons in the mtDNA circle are tightly packed, with no spacing introns. Mitochondrial DNA is histone-free, has limited repair ability, and therefore has a relatively high mutation-fixation rate (5–10 times that of scnDNA) [6]. Although mtDNA has evolved faster than the nuclear genome, the rate of evolution is different for different regions of mtDNA and has been used to examine various phylogenetic relationships. 12s rDNA is highly conserved and has been employed to illustrate phylogeny of higher categorical levels such as in phyla or subphyla. 16s rDNA is usually used for phylogenetic studies at mid-categorical levels such as in families or rare genera [7]. Compared to 12s and 16s rDNAs, the mitochondrial protein-coding genes

evolve much faster and are powerful markers for inferring evolution history in lower categorical levels such as families, genera, and species. This feature of mtDNA in phylogeny is suitable for resolving taxonomic uncertainties in conservation genetics. However, when mtDNA is used to determine species most deserving of protection, the following aspects should be considered: (i) Co-evolution between mitochondrial and nuclear genomes generates a lineage-specific mtDNA evolutionary rate [8, 9]. Correctly resolving taxonomic questions for this lineage requires modification of the molecular clock (hypothesizing that molecular change is linear with time, and constant over different taxa and in different places) and collection of mitochondrial data from other animal lineages. (ii) The level of efficiency of mtDNA repair mechanisms has been implicated in influencing substitution rates. The inefficient repair causes high rates of nucleotide substitution [4] and the valid repair mechanism leads to a low rate of evolution [10]. This mechanism yields special evolution of mtDNA in certain species or kinds of species. In this case, the correct taxonomic status of uncertain species will rely on nuclear DNA sequences. (iii) Although mtDNA gene regions have proven powerful in elucidating phylogenetic problems at the species level, some factors derived from intergenomic co-adaptation can quicken or slow the rate of base substitution at various mtDNA loci and cause evolutionary rate variations between sites [11]. Consequently, mtDNA sequences not less than two coding genes in size should be chosen to infer a concordant species tree (phylogenetic trees representing evolutionary relationships between different species) rather than a gene tree (phylogenetic trees showing the relationships between different copies of a single locus).

The mitochondrial CR is the major noncoding region of the animal mtDNA molecule. The vertebrate CR is commonly subdivided into three domains that differ from each other in base composition as well as in rate and mode of evolution [12]. The central domain of the CR, containing the heavy strand's origin of replication, is relatively conserved. In contrast, the two domains flanking the central domain (domains I and II) are typically hypervariable in base substitutions and indels (abbreviation of insertion/deletion mutation). Due to the fast rate of evolution of domains I and II, the CR has been typically deemed to be most appropriate for intraspecific studies [12]. Nevertheless, the two hypervariable domains have limited base substitution variation between individuals, *i.e.*, haplotypes (the combination of a set of linked loci on a given single stretch of DNA) can be shared by several animals, even in many individuals of an endangered species. This implies that recent loss of genetic variation leaves little trace on mtDNA, and therefore that the mtDNA CR is a powerful tool for determining the current status of population structure and identification of subspecies and species, rather than the individual identity and contemporary changes in genetic structure. On the other hand, a frequent feature of many, but not all, CRs is short tandemly repeated sequences. The vast majority of mtDNA size variation cases are a direct consequence of variable numbers of tandem repeats (VNTRs) in the CR [13]. Despite its sensitivity (it is able to show size polymorphism in the same individual), the use of mtVNTRs as a tool for population subdivision is in question because of problems such as poor reproducibility using different PCR or gel conditions, number-biased or size-biased alleles (an alternative form of a gene locus) and nonhomogeneous tissues [13].

A typical characteristic of mtDNA is maternal inheritance. The task of conservation genetics is not to examine genetic variation in populations and species but to set guidelines drawn from genetic data for ensuring scarce financial resources are channeled to the population/species most in need. Thus, efficient conservation strategies depend on neither maternal nor paternal variation, but heavily on biparental nuclear genetic variability, representing the characteristics needed to cope with environmental conditions. This approach determines that mtDNA is an auxiliary tool in conservation genetics.

The drawbacks of mtDNA in population genetics have been thoroughly discussed in a recent review by Zhang and Hewitt [5]. Here we attempt to deal with the limitations in applying uniparentally inherited mtDNA data to conservation genetics. In summary, mtDNA is the best tool for resolving taxonomic problems in conservation genetics. Nevertheless, one should be cautious with gene-specific, species-specific, and lineagespecific evolution in mtDNA. At the same time, the maternal inheritance of mtDNA restricts it to exploring events at the maternal angle and destines it to be a useful auxiliary marker to nuclear DNA. Lastly, it is worth noting that mtDNA is of little use in investigating recent loss of genetic variation and any individual-level events such as identity, individual dispersal, and mating systems.

3 Nuclear DNA markers

The focus of conservation genetics is on contemporary genetic structuring in small populations, and as such requires DNA markers that have a high evolutionary rate. VNTR markers are dispersed throughout the eukaryotic nuclear genome and their polymorphisms are the result of variations in the number of tandem repeats in a short core sequence (Fig. 1). VNTR markers have two main classes – microsatellites and minisatellites – both of which are usually characterized by a high degree of length polymorphism. Microsatellites are tandemly repeated

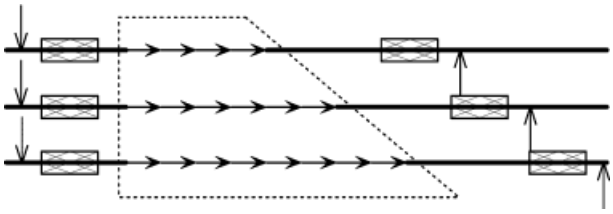

Figure 1. Polymorphisms in VNTRs. Vertical arrows represent restriction enzyme recognition sites; horizontal arrows are repeat units in VNTR; the dotted shape marks out core sequences of the VNTR loci; the bold lines indicate regions flanking core sequences; the shaded boxes show primers binding the flanking sequences.

motifs of 1–6 bases and can repeat from about 5–100 times at each locus. Minisatellites are tandemly repeating motifs of 8–100 bases that can repeat from two to several hundred times at each locus [15]. Microsatellites are more or less randomly scattered throughout the genome and frequently appear in transcription units. In contrast, minisatellites are interspersed but often clustered in telomeric regions. The VNTR loci in both mini- and microsatellites exhibit mutation rates several orders of magnitude higher than those of other DNA sequences, and are particularly suitable for analyzing recent historic events [16]. The differences both in length of repeat unit and in number of tandem repeats determines the different applications and limitations for these two kinds of VNTR markers.

3.1 Microsatellite DNA markers

Microsatellites are stretches of short DNA sequence in which a motif of one to six bases is tandemly repeated. With the advent of polymerase chain reaction (PCR) technology this property of microsatellite DNA was converted into a highly versatile genetic marker. PCR products of different lengths can be amplified with primers locating the relatively conserved flanking regions of microsatellites (Fig. 1). Microsatellites can be isolated from virtually any target species of interest since $10^4 - 10^5$ microsatellite loci are held in the genome [15]. Microsatellites are multi-allelic in a population and bi-allelic in an individual. They are inherited in a co-dominant Mendelian manner and can reveal heterozygote (with two different alleles at a locus) and homozygote (with two copies of the same allele at a gene locus) in each individual. The variability of microsatellites is often so high that even with a small number of loci and a large number of individuals, all individuals have a unique multilocus genotype (the combination of alleles an organism possesses). It is therefore possible to address issues such as discrimination, relationships, structure and classification, not only at the population (using allelic frequencies) but also at the individual (using genotype) level [17]. However, microsatellite polymorphism derived from variations in the number of tandem repeats is prone to the effects of sequence variation in the repeat unit and in the flanking region. Mutation in the repeat units and flanking regions results in practical and data problems in population and/or conservation genetics, particularly in wild populations of endangered animals.

The major drawback of microsatellites is that they need to be isolated *de novo* from most species being examined for the first time [18]. This is due to two facts: (i) Microsatellites are usually found in noncoding regions where the nucleotide substitution rate is higher than in the coding region. Consequently, the strategy of designing 'universal primers' matching conserved sequences is problematic. (ii) Apart from the variation in the number of repeats, nucleotide substitutions within the repeats are observed between species when employing the same primer pair [19]. These variations are species-specific or common to a genus or family and probably explain why loci are highly polymorphic in one species but monomorphic in another.

Microsatellite alleles generally correspond to DNA fragments of different sizes as revealed by electrophoretic methods. Microsatellite analyses assume that comigrating fragments are homologous, whereas there are few *a priori* reasons to assume this. In some cases, homologous alleles have been incorrectly scored as heterologous loci due to size heteroplasy (false inequality of alleles based on an increase in the number of nucleotides to different size) derived from the following two practical problems. (i) 'Slippage' (a mutation process whereby a simple sequence tandem repeat grows by addition or subtraction of the "beads" of simple units that make up the "necklace"). Although mutational mechanisms of microsatellites remain controversial, slippage mechanisms are putatively responsible for a large proportion of their variation. During the PCR amplification process the thermopolymerase can 'slip', leading to products that differ in size by approximately 1–5 repeat units from the expected product [20]. Such replication slippage can be a significant problem when analyzing mono- and dinucleotide repeats. When the products of a heterozygous individual overlap the slippage products it becomes particularly difficult to differentiate between true and unwanted products [20]. (ii) Inaccurate allele identification may be caused by the tendency of *Taq* polymerase to add an adenosine nucleotide to the 3'-end of the amplified product [21]. Although not such a problem if the extra nucleotide is always or never added, errors may occur in size determination if the extra nucleotide is only occasionally added.

In regions flanking repeat stretches, insertion/deletion events involving multiple nucleotides (1–5) often occur with high frequency [19]. The indels will change the length of an amplified product, which may then comigrate with an allele without indels. This seeming homology may be misinterpreted as different length of the tandem repeats and result in a misidentification of the alleles if only the allele size was measured. This is called size homoplasy (false equality of alleles based on independent mutation to the same size) of microsatellites. The size homoplasy cannot provide real genealogical information on the evolutionary history. Thus, this is the greatest problem facing the use of microsatellites in phylogenetic analyses.

Apart from indels, point mutations frequently occur in flanking sequences. Although point mutations may not change the length of a microsatellite product, mutations in the binding region of one or both of the microsatellite primers can inhibit annealing that may result in the reduction or loss of the PCR product [21]. Such products are termed null alleles (allele that fails to become visualized under the analytical conditions). The direct consequences of null alleles are fewer heterozygotes than expected in a randomly mating population, making them particularly responsible for mismatch between parent-offspring pairs [22]. The use of heterologous primers is likely to increase the incidence of null allele detection. In cross-species studies it is likely that as the taxonomic distances between taxa increase then the incidence of null alleles will also increase. Nevertheless, in some cases the high proportion of null alleles (30% of loci used) may be the result of using homologous primers [21].

Microsatellites constitute a rather large fraction of noncoding DNA and are relatively rare in protein-coding regions [23, 24]. The diverse distribution of microsatellites determines different selective features (neutral, referring a mutation has no effect on the fitness of the organism, or non-neutral), causing the mode and tempo of evolution to vary greatly among loci and taxa [25]. This implies that (i) the non-neutral evolution of some microsatellite loci may result in gene history rather than real ancestral information (species tree), and (ii) there is a clear need to perform equilibrium tests to select neutral microsatellite markers for genetic analyses.

The above-mentioned practical problems can be overcome by controlling workable conditions. Uncertain size homoplasy data and biased analyses of non-neutral mutations in some microsatellites may result in incorrect phylogenetic histories, indicating caution is required when employing microsatellite markers in population genetics. Both size homoplasy and null alleles lead to an underestimate of genetic diversity but will not cause significant biases in genetic analyses because the large amount of variability at microsatellite loci often makes up for these shortcomings [17]. However, it must be remembered that such genetic compensation depends on abundant variation of microsatellites, which may be problematic in endangered species. As mentioned in the introduction, conservation genetics focuses on small and/or declining populations. The multi-allelic state of microsatellites derives from accumulated variations in a population. When a population is driven to the brink of extinction and held there for several generations, a large proportion of that species' neutral variability (mutations that have no phenotypic effects) may be eroded by chance loss of alleles. Examination of genetic variation over space and time has attracted increasing attention from conservation geneticists [26–31]. Such studies revealed that loss of alleles over generations was significant in small populations [26, 29, 31], suggesting some polymorphisms were rapidly wiped out by genetic drift in a short time. The statistical power of microsatellite markers depends on the number of loci used, the degree of polymorphism of each locus and the sample size [18]. Generally, a relatively low number of microsatellites with high polymorphism (often less than 20, sometimes less than 10) are available in population genetics [18]. Studies show 87.5% of endangered animals have less than 5 alleles per locus on average, whereas this is the case in only 19.2% of non-endangered species [1], indicating poor polymorphism of microsatellites in endangered species. That few microsatellites are used and there is low polymorphism per locus imply the power of microsatellite markers in large captive and/or wild populations is limited due to loss of many polymorphic loci. Taking captive pandas as an example, a set of microsatellite markers which had been successfully used for performing paternity testing between potential fathers and offspring were subsequently limited in their use following a validation study. That is, fathers were correctly assigned in only one-third of cases when unknown parents and babies from several families were purposely incorporated into a relatively large breeding population of giant panda (Yu, personal communication; unfortunately this finding has not been published because it is a negative result). This suggests microsatellites are useful in paternity analysis of a small group rather than a population. In this case, size homoplasy and null alleles at least partly contributed to the problems of microsatellite analyses. These observations show that caution should be used when employing microsatellites in endangered species studies.

The microsatellite technique is PCR-based, providing new opportunities for conservation genetics research when the amount of DNA is limited (*e.g.*, shed hair) or contaminated DNA (*e.g.*, feces). It offers the opportunity to obtain genetic samples in situations where traditional methods for tissue

or blood sampling are impractical. While the advantages provided by these opportunities are obvious, they also introduce two types of errors. (i) Allelic dropout. The preferential amplification of only one of two alleles is the most common and troublesome error associated with low concentration template DNA, such as that from shed hair and feces [32]. When allelic dropout occurs, a true heterozygote appears to be a homozygote, resulting in mistyping at the individual level, and an excess of homozygous genotypes at the population level. (ii) The second type of error is termed misprinting, which is particularly associated with feces analysis [33]. Misprinting is the amplification of false alleles resulting from small amounts of contaminating DNA in competition with similarly small amounts of template DNA. This genotyping error creates several genotypes per individual. Dropouts and misprinting have been found in essentially all studies based on noninvasive samples with mean rates of dropout ranging from 0.00–0.39 and misprinting from 0.003–0.11 per allele tested [34]. As a result, a multiple-tube method (two replications for heterozygous loci and seven replications for homozygous loci) was recommended [35]. However, the pervasive contamination may persistently give unreliable results, so that these genotyping errors still lead to highly-biased estimation (up to 5.5-fold) of population size despite using the multiple-tube approach [34]. When dealing with DNA from noninvasive samples, a single error in a multilocus genotype can create a false result. Therefore, the microsatellite loci amplified from faecal samples may present false individual identity and thus affect nearly all genetic analyses of natural populations despite theoretical feasibility. To solve these two types of problems, two aspects deserve attention: (i) it is essential to optimize the methods for storing feces and also for extracting DNA from feces. (ii) According to potential sources of contaminated DNA, species-specific microsatellite loci should be isolated.

3.2 Oligonucleotide fingerprinting

The two major disadvantages weakening the power of the microsatellite techniques are size homoplasy and null alleles, which result from mutations (base substitutions and indels) in the flanking regions of microsatellite core sequences. Justifiably, from a hybridization point of view, a VNTR probe designed from core sequences will avoid these limitations. Molecular hybridization is based on restriction fragments. The restriction fragment length polymorphism (RFLP) derives from three types of mutations: (i) the presence or absence of a specific restriction enzyme recognition site in the flanking regions of tandem repeat sequence stretches; (ii) insertions/deletions in the flanking sequence of VNTR loci; (iii) variability in length of VNTR loci. Traditional RFLPs are visualized by a probe specific for a locus in the VNTR loci flanking regions. In this case, the locus-specific probe detects the polymorphism between two homologous chromosomes of an individual and thus is able to identify homozygote and heterozygote, similar to single microsatellite loci. Nevertheless, in contrast to the variation in length of VNTRs, the locus-specific probe requires changes of recognition site of the restriction enzyme selected at the flanking sequences of VNTR loci. So, the customary RFLPs provide insufficient data for precise analyses. In 1985, Jeffreys *et al.* [36] first developed individual-specific DNA fingerprints by using DNA probes containing tandem repeats of a core sequence to hybridize multiple variable DNA fragments. Subsequently, Epplen and co-workers [37, 38] used synthetic tandem repeats as probes, rather than cloned natural tandem repeats, to perform molecular hybridization and also obtain individual-specific DNA banding patterns, and thus created a new technique called oligonucleotide fingerprinting. The advantages of using oligonucleotides compared to DNA fingerprinting include: (i) Stability of chemically synthesized oligonucleotides ensures concordant hybridization results between different laboratories. (ii) In-gel hybridization with oligonucleotide probes is simple (does not require Southern blotting) and fast (one-sixth the exposure time compared with classical minisatellite probes). (iii) Nonradioactive fingerprinting can be performed with sufficient sensitivity using digoxigenated oligonucleotides [39]. (iv) Under appropriate hybridizing conditions, oligonucleotide probes are absolutely specific such that a single base mismatch will obstruct hybridization [37]. (v) Chemically synthesized oligonucleotide probes have proven useful in individualization and genetic relationship studies in previously completely uncharacterized species [40].

As a probe-based technique, oligonucleotide fingerprinting avoids all PCR-induced practical and data problems inherent in microsatellite marker use. Nonetheless, it has intrinsic drawbacks common to all molecular hybridization techniques, such as multiple steps (relatively time-consuming) and requirement for a relatively large amount of DNA. Although the need for a large quantity of DNA suggests DNA fingerprinting may be unsuitable for conservation genetics, we have employed oligonucleotide fingerprinting to solve many conservation genetics issues for endangered animals [41–45]. Formalin-fixed tissues and feces are good DNA sources for endangered species but are rarely used for DNA fingerprinting due to problems of DNA extraction. However, our laboratory has developed efficient approaches for isolation of DNA from formalin-fixed and faecal samples [44, 46], and such methods create an avenue for DNA fingerprinting in conservation genetics.

Previous studies revealed that oligonucleotide probes consisting of 15–30 nucleotides produced good and specific hybridization patterns [47]. Thus, the whole length of an oligonucleotide probe synthesized (*i.e.*, multiplying the number of nucleotides in a repeat unit and the number of tandem repeats) should be within this range (15–30), suggesting that the number of tandem repeats in a VNTR locus is of no importance to design an oligonucleotide probe. According to this rationale of the probe design, some oliogonucleotide probes were successfully developed [48–51]. Oligonucleotide probe sequences correspond to tandem repeat units in microsatellites and minisatellites, and are therefore classified as oligonucleotide microsatellite probes such as $(CAC)_5$ [39] and $(GT)_8$ [48], and oligonucleotide minisatellite probes such as $(CTCCACCT)_3$ [49]. The mutation rate of microsatellites is very high, 10^{-2}–10^{-6} events per locus per generation (some loci have 100% mutation rate) [48]. The application of microsatellite analysis to population genetics requires caution as the evolutionary time frames covered in population genetics are often too long to allow novel microsatellite mutations to be ignored [52]. Therefore, we recommend relatively stable oligonucleotide minisatellite probes be used in conservation genetics. The individual-specific DNA "fingerprints" produced by oligonucleotide probes provides a powerful method for individual identification, paternity testing and potential applications in natural populations. To reach the full potential of oligonucleotide fingerprinting, it is essential to exclude hybridization of the VNTR probe to contaminated DNA in feces. One strategy to remove obstacles is to develop species-specific but individualpolymorphic probes. The species-specific probe, however, produces an identical DNA banding pattern in every individual and is of limited use for identifying species [44]. Another strategy is to reduce the amount of contaminated DNA to a very small proportion of total DNA. The approach we published [44] is based on repeated centrifugation to remove minor components (*e.g.*, bacteria and plant remnants) from the faecal sample thus ultimately enriching the DNA from the sloughed intestinal cells of the target species. This method works well and has been successfully used to determine population sizes in wild giant pandas in two reserves (Wan, unpublished results).

The high variability of minisatellites allows them to be used for investigating rapid loss of genetic variation, population structure, and paternity or parentage analysis. On the other hand, the correct individual-specific DNA fingerprints from a natural population ensure the power of oligonucleotide fingerprinting to estimate population size, individual relatedness, mating system, population subdivision, *etc.* Here, we intend to emphasize two unique applications of oligonucleotide minisatellites, namely reconstruction of population history and identification of contemporary gene flow between fragmented subpopulations. (i) Minisatellite polymorphism visualized by southern hybridization avoids the distortion of phylogeny resulting from size homoplasy in PCR-derived microsatellites [53]. The mutation rate of minisatellites is 100–1000 times higher than scnDNA sequences [54]. Therefore, the relatively constant genetic data on a fine time scale are particularly suitable for inferring recent population history. Taking mtDNA as a control, it is possible to deduce a valid time span for this population history. The pioneering studies in estimating divergence time of species revealed that mtDNA can reflect species evolutionary history back at least 1 million years [55–59]. As the rate of mtDNA evolution is 5–10 times faster than scnDNA (minisatellite is 100–1000 times), we conservatively estimate that DNA fingerprinting can be used to determine population histories within the last 100 000 years. Additionally, if a particular allele shared by a group, population or meta-population but not found elsewhere was observed in neutral DNA fingerprints, it can be used for provenance identification, which provides strong ancillary evidence for the inferred population history. A new subspecies of the giant panda, resulting from the glaciation 10 000 years ago, has been revealed by oligonucleotide fingerprinting [45]. (ii) Human activities have fragmented wild populations into isolated populations, which have been subjected to more recent fragmentation into unequal subpopulations. While historic gene flow between populations isolated for a long time is universally estimated by indirect *F*st estimators, gene flow between more recently separated subpopulations is unknown. Individual-based analyses potentially allows measurement of contemporary gene flow between subpopulations. Gene flow between two subpopulations is evident when parentage testing based on DNA fingerprints shows parents and babies are present in different subpopulations.

4 Issues for conservation genetics

Effective decision-making is crucial in this era of pragmatic conservation where wildlife managers govern the likelihood of a species' survival. Conservation geneticists develop gene maps of target species so managers can chart the most effective means of preserving animal genetic diversity. Conservation genetics helps managers protect biodiversity by identifying a series of conservation units, including species, evolutionarily significant units (ESUs), management units (MUs), action units (AUs), and family nets (FNs). Undoubtedly, species clearly require conservation as separate units. However, taxonomic problems often rise in the following cases when defining species priority: (i) The taxonomic status of many taxa,

especially invertebrates and lower plants, is frequently unknown [1]. Unrecognized endangered species may inadvertently be allowed to become extinct. (ii) An apparently widespread and low-risk species may, in reality, comprise a complex of distinct taxa [60]. Incorrect incorporation of several distinct species into one recognized species may deny protection of endangered species. (iii) Interspecific hybridization creates problems in conservation. Hybridization includes ancient events and recent hybridization. If the hybridization is an ancient event, whether these ancient hybrids are worthy of conservation relies on the current status of the two ancestral species [61, 62]. If it is recent hybridization, an effort should be made to monitor the extent of hybridization between rare species and widespread or introduced species and to prevent continued hybridization [63]. The former two approaches require genetic data from mtDNA coding genes [64–67]. In contrast, the last approach requires both mtDNA and nuclear genetic information to clarify the extent and location of hybrid populations, the directionality of hybridization, and the degree to which taxon-specific alleles have penetrated into the range of another taxon (so-called introgression) [68–70]. Molecular markers assist in resolving taxonomic uncertainties so that endangered species are not denied protection, nor wasted on abundant species.

In prehistory many species were separated by ice, desert, or ocean over periods that allowed for compensatory adaptation and genetic isolation. If these populations within species show significant adaptive differentiation to different habitats (ecological niches), or significant genetic differentiation, then they may justify management as separate evolutionary lineages, termed ESUs [1]. The ESU was developed to provide an objective approach to prioritizing units for protection below the taxonomic level [71]. An ESU can probably be considered as a subspecies on the path to speciation. Subspecies classification is often controversial and requires morphological evidence, so ESUs ensure the conservation of potential subspecies. Despite various definitions, a uniform agreement might be that an ESU is a lineage demonstrating highly restricted gene flow from other such lineages within the higher organizational level (lineage) of the species [72]. ESUs have been distinguished from other populations in some species based on mtDNA [73] and/or nuclear DNA [73, 75]. However, there were large discrepancies between such inferred ESUs depending upon whether information was obtained by mtDNA or nuclear markers [74, 75]. This discordance stresses the importance of collecting data from both types of genetic markers before interpreting data and making recommendations for the conservation and management of natural populations. It is worth pointing out that in previous studies both mtDNA and nuclear DNA were used to identify ESUs by choosing loci and analyzing the data by statistical methods. The locus-biased selection to a large part affects the inference of correct ESUs. Even when using the mtDNA genome the characteristic of maternal inheritance still possibly causes biased results for the whole species. DNA fingerprints derived from different subspecies/species of fish [76], bird [77], and mammal [78], revealed that large differences in the distribution range of bands, the average number of bands per individual, and specific homozygous alleles exist not only inter-species but also inter-subspecies. Because of ESU at the subspecies level, DNA fingerprinting which detects all alleles of the VNTR probe in the whole genome is promising in discriminating ESUs of endangered species. By simply observing the distribution range of bands and specific homozygous loci, two ESUs and three MUs were successfully distinguished in the giant panda population, and were validated by statistical methods [45].

Human activity or feral invasions have caused catastrophic fragmentation of many populations. Decades later, geneticists are left to puzzle over whether a discrete group of animals is isolated from a larger population by historical factors or contemporary human invasion. The DNA marker system provides a highly accurate tool for establishing links with possible parent populations. Populations which reveal less phylogenetic separation than reciprocal monophyly by genetic analyses have been defined as MUs [79]. The MU was intended to be a level of conservation unit below that of the larger ESU that had statistically significant divergence in allele frequencies (nuclear or mitochondrial). Before setting an ESU, clearly there is a need to explore population history and to infer demographic forces such as geographical barriers, glaciation, ecological shifts and other factors. In contrast to ESUs derived from historical genetic differentiation, the focus of the MU is on contemporary population structuring and short-term monitoring rather than historical factors, requiring a determination of recent genetic structure, dispersal and migration patterns of current fragmented populations [80].

MUs do not reveal detailed genetic management to wildlife managers but indicate these populations should be treated as a unit. The precise results of genetic detection are vital under many circumstances, including the following: (i) Determining loss of which population will result in a catastrophic loss of genetic diversity, and thus to which population scarce financial resources should be channeled. (ii) If a species survives in small, scattered populations that have no contact, should attempts be made to expand smaller groups with introductions from larger populations? Little genetic variation between populations, suggesting compatibility with each other, is useful

to rescue the species. However, if each population shows significant genetic differences, merging the gene pools of two populations may result in an overall loss of genetic diversity for the species. (iii) Captive populations have been implemented for re-introduction and release to expand endangered species [81, 82]. However, before carrying out re-introduction and release plans, the status of genetic diversity of candidate populations (*i.e.*, historic populations in the case of re-introduction) must be investigated to identify appropriate sites where populations have similar genetic variability to breeding ones. We refer to these populations needing immediate manmade protection plans as AUs.

Small and isolated populations are prone to extinction due to environmental stochasticity (*e.g.*, natural catastrophes and disease), demographic stochasticity (*i.e.*, random variations in sex ratio, mortality or reproduction), genetic effects (*e.g.*, mutation accumulation and inbreeding), and too little immigration [83]. As for the first factor, we have no countermeasures other than increasing genetic variation by swiftly elevating population size. Molecular markers in the DNA of all individuals provide highly accurate tools for assessing population size, sex ratio, mortality, reproduction, effective population size, inbreeding, and migration and are thus very useful in monitoring population growth or decline. According to this genetic information, effective strategies can be implemented to stop or at least slow the process of extinction resulting from these causes. Additionally, dispersal and migration patterns inferred from family relationships of currently fragmented populations are useful for evaluating the population's status, and identifying the most endangered populations in the wild in order to allocate resources. However, the premise of these efficient countermeasures is to precisely identify individual identity and family relationships, which we term the FN, from fecal samples. The use of oligonucleotide fingerprinting and faecal DNA will provide unprecedented opportunities for conservation genetics.

5 Selection of molecular markers in conservation genetics

The process of choosing a marker system is perhaps the most critical step in conservation genetics analysis, as selection of inappropriate molecular markers will result in incorrect conservation actions. The aforementioned properties of nuclear DNA and mtDNA markers show that scnDNA has the slowest evolutionary rate and VNTR the fastest, 100–1000 times than of scnDNA. MtDNA evolves about 5–10 times faster than scnDNA, where coding genes are conserved and the D-loop region is hypervariable. Thus, different DNA signatures are left on different types of molecular markers (Fig. 2). The data presented in Fig. 2 show that both single-copy nuclear DNA (scnDNA) and mtDNA contain historic genetic mutations and are suitable for resolving taxonomic uncertainties; mtDNA markers and VNTR contain more information on recent genetic variation than scnDNA; VNTR is the best choice for inferring contemporary genetic patterns. The identification of ESU depends on significantly differentiated genetic structure detected by presumably neutral markers. However, the genetic structure revealed by multiple classes of DNA markers shows that founder effects/bottlenecks [84, 85] and sex-biased dispersal [86, 87] may result in significant differentiation which may cause mis-identification of ESUs. A partial bottleneck may be caused by environmental factors such as disease epidemics and historical isolation. The distinct genetic unit is not an ESU unless the bottlenecks are accompanied by glaciation or ecological shift, which can result in adaptive differences. Regarding sex-biased dispersal, if all populations show this characteristic, it suggests historic mating patterns of target species that are useful for designing conservation strategies [83, 88]. If this phenomenon appeared in one population, it does not imply an ESU but an unusual dispersal pattern. This distinct population should be considered an MU or an AU. In contrast to an ESU's historic genetic variation, MUs display

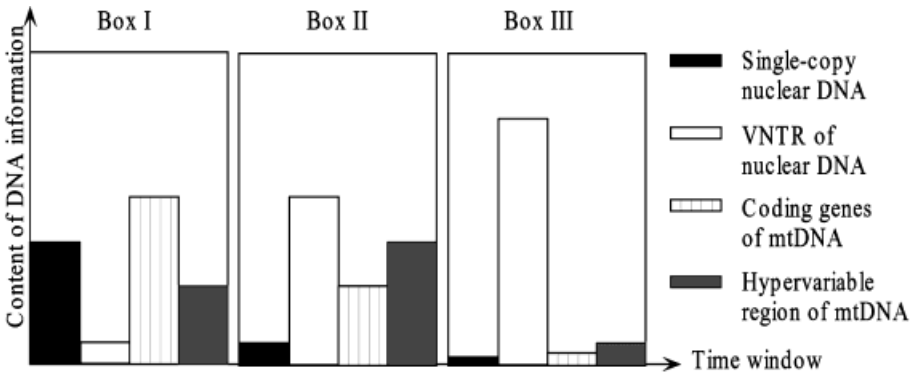

Figure 2. Different genetic variations recorded in different time windows. Box I shows historic genetic variation corresponding to a time window of speciation. Box II records recent events and represents a time window of differentiation of ESUs. Box III corresponds to contemporary genetic patterns and shows a time window defining MU, AU, and FN.

contemporary genetic structuring, while AUs and FNs reflect genetic patterns of living populations in the wild and/or captivity. Taking diverse requirements of conservation issues into account, mtDNA and nuclear VNTR markers may be employed for different issues in conservation genetics (Table 1).

Table 1. Selecting DNA markers for identifying different conservation units

Marker types	Species	ESU	MU	AU	FN
Coding genes of mtDNA	√	√			
CR of mtDNA	√	√			
PCR-based microsatellites		√	√	√	
Oligonucleotide probes		√	√	√	√

When utilizing mtDNA to define species priority, one should use multiple loci of authentic mtDNA rather than a single gene in order to infer correct species identity. If necessary (to avoid species-specific evolution of mtDNA), nuclear markers can be identified to supply additional support to the species identity. Because unrelated phenomena are likely to leave their footprints on genetic structure, clearly there is a need to combine several kinds of markers to identify a true ESU. Although size homoplasy and null alleles affect PCR-based genetic analyses of microsatellites, it is not a large problem when comparing genetic structure among populations due to the similar degree of detection biases. Microsatellites are useful for paternity testing in breeding populations due to the very small sample size (offspring and potential fathers). However, when analyzing a natural population of endangered species, especially of critically endangered species, many problems can arise from PCR-based microsatellite assays. Firstly, high homozygosity (the number of individuals heterozygous for a locus divided by total number of individuals sampled) in endangered species largely reduces the number and level of variability of microsatellite loci [1], which further stresses estimation biases of size homoplasy and null alleles. Secondly, to avoid the influence of contaminated DNA, species-specific primers for microsatellites should be designed. However, it seems it may be impossible to comply with the conflicting requirements for primer sequences to be evolutionarily conserved but present high intraspecific variability in core sequences of microsatellites [5]. Lastly, microsatellite techniques are always used in cases of low DNA amount, which generally precludes a multiple-tube PCR approach to exclude errors of allelic dropout. These limitations lead to strongly biased results and misdirect the design of conservation programs. In striking contrast to PCR-based microsatellite analysis, an oligonucleotide probe rejecting a single base mismatch reveals the polymorphism of the whole genome rather than certain loci. The abundant polymorphic loci generate individual-specific fingerprints and provide a highly accurate tool for genetic analyses of endangered species. However, in some cases only a limited DNA amount is available (*e.g.*, endangered insects), which does not favor oligonucleotide fingerprinting. Even though PCR-based microsatellite assays are used to overcome this obstacle, the above-mentioned intrinsic drawbacks may result in incorrect results. These cases present conservation geneticists with a dilemma.

In conclusion, oligonucleotide fingerprinting provides a reliable measure of genetic variation over relatively recent periods of time and should be used firstly in practicable environments when defining MUs, AUs, and FNs. In some cases that are not suited to fancy fingerprinting, microsatellite assays can be adapted instead if abundant polymorphic loci are available. When resolving taxonomic problems, mtDNA is the best choice. Nonetheless, if designing ESUs, mtDNA, oligonucleotide fingerprinting and micosatellites should be utilized together.

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