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Distances and their visualization in studies of spatial-temporal genetic variation using single nucleotide polymorphisms (SNPs)

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Running Title: SNPs and genetic distances

Abstract

1. Distance measures are widely used for examining genetic structure in datasets that comprise many individuals scored for a very large number of attributes. Genotype datasets composed of single nucleotide polymorphisms (SNPs) typically contain bi-allelic scores for tens of thousands if not hundreds of thousands of loci.
2. We examine the application of distance measures to SNP data (both genotypes and sequence tag presence-absence) and use real datasets and simulated data to illustrate pitfalls in the application of genetic distances and their visualization.
3. Missing values arise from ascertainment issues in the SNP discovery process (null alleles in the case of SNP genotyping; true missing data in the case of sequence tag presence-absence data). Missing values can cause displacement of affected individuals from their natural groupings and artificial inflation of confidence envelopes, leading to potential misinterpretation. Failure of a distance measure to conform to metric and Euclidean properties is important but only likely to create unacceptable outcomes in extreme cases. Lack of randomness in the selection of individuals (e.g. inclusion of sibs) and lack of independence of both individuals and loci (e.g. polymorphic haploblocks), can lead to substantial and otherwise inexplicable distortions of the visual representations and again, potential misinterpretation.
4. Euclidean Distance is the metric of choice in many distance studies. However, other measures may be preferable because of underlying models of divergence, population demographic history, linkage disequilibrium, because it is desirable to down-weight joint absences, or because of other characteristics specific to the data or analyses. Distance measures for SNP genotype data that depend on the arbitrary choice of reference and alternate alleles (e.g. Bray-Curtis distance) should be avoided. Careful consideration should be given to which state is scored zero when applying binary distance measures to fragment presence-absence data (e.g. Jaccard distance). Filtering on missing values then imputing those that remain avoids distortion in visual representations. Presence of closely related individuals or polymorphic haploblocks in the genomes of target species with limited genomic information occasionally emerge as challenges.

Keywords: Principal Components Analysis, Principal Coordinates Analysis, Genetic distance, Metric distance, Euclidean distance

Introduction

Population genetics is the study of the interplay of genetic drift, gene flow, recombination and selection, and to a lesser extent mutation, as they come to influence the contemporary genetic composition of populations. Finite populations can be expected to vary in genetic composition in space and through time under the influence of genetic drift alone. Rate of genetic drift strongly depends on overall population size and the level of spatial sub-structuring within populations. Populations are not static, and the history of population size fluctuations has a bearing on contemporary patterns of divergence evident in distance analyses, particularly if there has been a recent reduction in size or a sustained bottleneck. Divergence in allelic profiles can be reinforced and accelerated (or in some cases, impeded) by local selection (A. W. F. Edwards & Cavalli-Sforza, 1967). The effect of these evolutionary processes on genetic distance are moderated by demographic events such as gene flow between populations of the same species, and hybridization and introgression between closely related species. Mutation sustains allelic variation in the context of allelic loss through drift and/or selection, but its effects are likely to be small compared to other influences in the context of population genetics (A. W. F. Edwards & Cavalli-Sforza, 1967).

Many measures of genetic similarity and dissimilarity have been developed to quantify inter-individual and inter-population variation. The concept of genetic distance (Sanghvi, 1953) is now a fundamental tool in genetics (Nei & Kumar, 2000). Genetic distances fall into several broad classes. Some are obtained by direct measurement, such as immunological distance (Faith, 1985) or DNA-DNA hybridization (de Ley et al., 1970; Hirayama et al., 1996; Kirsch et al., 1990). However, most genetic distances are calculated from character states (genotypes) arranged as a matrix of individuals (as entities) by attributes (genetic loci). Genetic distances can be further classified by whether they will be used to infer patterns of ancestry and descent among species (phylogenetics), the structure and relationships among populations of a species at various scales of divergence with or without gene flow (population genetics), or relationships among individuals (e.g. kinship).

Even if the focus is on elucidating contemporary divergence among populations, genetic distances between populations and the genetic distances between the individuals that comprise those populations are interdependent. In this paper, we deal with distances defined for both individuals and populations, in the broader context of divergence among populations and subpopulations.

The array of available measures of distance and similarity/dissimilarity (Deza & Deza, 2009) is daunting. In this article, we specifically address analyses of single nucleotide polymorphisms (SNPs), markers that are commonly used in studies of spatial or temporal variation among individuals and populations of a species or closely related species. SNPs have particular characteristics that influence the choice of a distance measure. SNPs can have more than two alleles (i.e. are multiallelic) but in practice, sites with more than two allelic states are filtered out during the selection of markers as part of pipelines to eliminate non-homologous sequence tags. Fortunately, multiallelic SNP sites are rarely observed. As a result of this filtering, SNP markers are bi-allelic, typically scored as the frequency of the alternate allele – 0 for homozygous reference allele, 1 for heterozygotes, and 2 for homozygous alternate allele (in diploid organisms). Such biallelic markers have characteristics that substantially limit options for a distance measure. For example, the values of 0 and 2 carry equal weight because the choice of reference allele and alternate allele is arbitrary. Any distance measure that gives differential weight to joint zeros (e.g. Bray & Curtis, 1957 Distance) will yield values that depend on the arbitrary choice of which allele is reference and which is alternate at each locus. Such distance measures can be eliminated from options available for SNP genotype data. Standardization or normalization across attributes (loci) is not required because the attributes (loci) are all measured as genotypes on the same scale (0, 1 or 2); this is not the case under some circumstances in multiallelic systems. That said, the biallelic nature of SNP markers permits the easy calculation of the maximum distance possible, which permits scaling distances to fall conveniently in the range of [0,1] while maintaining comparability across studies. Finally, issues that arise in a multiallelic context do not apply in a biallelic context. For example, Rogers D (Rogers, 1972), and therefore Standard Euclidean Distance, can yield undesirable results when two populations are both polymorphic at a site, but share no alleles (Nei & Kumar, 2000:246). This situation does not arise in the biallelic case, and so Standard Euclidean Distance (or Rogers D) is often the distance of choice in SNP studies for both individuals and populations.

A second form of informative genetic data comprises presence or absence of the sequence tag typically denoted as 1 for presence and 0 for absence, hereafter referred to as SNP PA data. Unlike the co-dominant SNP genotype markers, these are dominant markers. Absence of a sequence tag in this case is not because of missing data *per se* (absence of evidence), but instead is the true absence of sequence tag in a sample (evidence of absence). Technical artefacts such as low DNA quality or quantity, or failure of experimental protocol

resulting in shallow sequencing depth, may occasionally lead to the missing data owing to the absence of evidence. Typically, this is overcome by quality control processes (validation of Mendelian inheritance consistent with dominant markers), and data filtering is routinely performed (principally on read depth and consistency of read depth across individuals) to distinguish between the true absence and missing data for sequence tags. Therefore, absence of a sequence tag is assumed to arise as a null allele, which signifies a mutation at one or both of the restriction enzyme recognition sites. Provided technical reproducibility is achieved in the generation of data, SNP presence-absences are valid genetic markers in their own right. Since SNP PA data are binary data, many binary distance measures (Deza & Deza, 2009) have been co-opted for genetic studies of SNP PA data (e.g. Jaccard Distance).

In this paper, we first consider genetic distances commonly used in analyses of SNP data (both SNP genotypic data and SNP PA data) as they apply to individuals and populations. Although genetic distances are used in a wide range of contexts (Jansen & van Hintum, 2007; Libiger et al., 2009; Yin, 2020), we focus in this paper (not exclusively) on the application of distances in studies of spatial and temporal genetic structure among populations where allelic profiles are governed principally by recent or contemporary processes of drift, selection and gene flow. We do not consider distance measures used to reveal deeper historical patterns of ancestry and descent among lineages on independent evolutionary trajectories where the pattern of mutational events dominates (species-level phylogenetics). Nor do we consider distance measures used to quantify distances between pairs of SNPs as opposed to pairs of individuals or populations (Müller et al., 2005).

We second examine approaches for visualizing distances in multivariable space, in particular, Principal Components Analysis (PCA) (Hotelling, 1933; Jolliffe, 2002; Pearson, 1901) and Principal Coordinates Analysis (PCoA) (Gower, 1966) collectively referred to as ordination. These techniques have found application in many diverse fields such as ecology, economics, psychology, meteorology, oceanography, human health and genetics as a descriptive and an exploratory tool to generate hypotheses for further examination (Jolliffe & Cadima, 2016) rather than a formal statistical analysis (but see Patterson et al., 2006).

Finally, we examine some practical aspects of applying distance analyses and their visualization. With the relatively recent advent of large SNP matrices, many applications of ordination examine spatial and temporal population structure in SNP datasets. We pay particular attention to underlying assumptions of the application of distance analyses and

visualization of spatio-temporal structure using ordination, which are poorly appreciated. These assumptions include the properties of various distance measures and the importance of metric and/or Euclidean properties for visualization, the impact of missing values and the importance of randomness in sampling and independence of individuals, their genotypes and the loci selected for screening. We briefly review the most commonly used distance measures as they apply to SNP datasets and their usefulness in analysing population structure.

Box 1. Glossary of terms

Population. A group of individuals of the same biological species that by virtue of occupying a well-defined geographic provenance, have opportunity to interbreed. Changes in the allelic profiles of a subset of individuals will come to affect the whole.

Sub-population. A subset of individuals of a population, occupying a well-defined geographic provenance and which, because of resistance to geneflow arising from partial or episodic isolation, have a distinct allele frequency profile. For ease of communication, we refer to populations on the implicit understanding that the point also applies also to subpopulations.

SNP. Single Nucleotide Polymorphism. A single base pair mutation that is polymorphic.

SNP data. Generic term for SNP genotype data and SNP PA data scored across multiple loci for each individual.

SNP genotype data. SNPs scored across multiple loci for each individual.

SNP PA data. Presence or Absence of the amplified sequence tags across multiple loci for each individual. Absences are typically "null alleles".

Entities. A collective term to refer to the samples, subjects, individuals, or populations from which the genotypes are scored.

Attributes. A collective term for the characters that are scored for each entity, in this case the SNP loci.

State. Refers to the value of an attribute (locus) for a given entity (individual) in the data matrix.

Distance. Term used loosely to encompass distance and dissimilarity measures (Gower & Legendre, 1986).

Euclidean distance. Metric distance that further can be represented faithfully in a space defined by Cartesian coordinates.

Metric distance. Distance that satisfies the formal metric properties – positive definite, symmetric, and conforms to the triangle inequality.

Dissimilarity measure. A quantitative measure of how different two individuals or populations are. Does not necessarily conform to the properties of a metric.

PCA. Principal Components Analysis, a classical ordination technique drawing upon the covariance matrix (unstandardized) or the correlation matrix (standardized) for the entities by attributes matrix.

PCoA. Principal Coordinates Analysis, a generalization of PCA that admits a wider range of distance and dissimilarity measures.

Reference [Alternate] allele. The allele, typically the most frequent or major allele, that is arbitrarily chosen as reference to distinguish it from the alternate allele.

MDS. Multidimensional scaling whereby individuals are represented in a Cartesian space of a specified dimension by iteratively matching the distances in that space with those of the associated distance or dissimilarity measure. It can be applied to data converted to ranks.

Methods

For the purposes of illustration, a dataset was constructed from a SNP matrix generated for the freshwater turtles in the genus *Emydura* (Georges et al., 2018), a recent radiation of Chelidae in Australasia. The dataset includes populations that vary in level of divergence to encompass variation within species and variation between closely related species. Sampling localities with evidence of admixture between species were removed. Monomorphic loci were removed, and the data was filtered on call rate (>95%), repeatability (>99.5%) and read depth ($5x$ < read depth < $50x$). Where there was more than one SNP per sequence tag, only one was retained at random. The resultant dataset had 18,196 SNP loci scored for 381 individuals from 7 sampling localities or populations – *Emydura victoriae* [Ord River, NT, n=15], *E. tanybaraga* [Holroyd River, Qld, n=10], *E. subglobosa worrelli* [Daly River, NT, n=25], *E. subglobosa subglobosa* [Fly River, PNG, n=55], *E. macquarii macquarii* [Murray Darling Basin north, NSW/Qld, n=152], *E. macquarii krefftii* [Fitzroy River, Qld, n=39] and *E. macquarii emmotti* [Cooper Creek, Qld, n=85]. The missing data rate was 1.7%,

subsequently imputed by nearest neighbour (Beretta & Santaniello, 2016) to yield a fully populated data matrix. The data are a subset of those published by Georges et al. (2018) for illustrative purposes only.

The above manipulations were performed in R package dartR Version 2.0.3 (Gruber et al., 2018; J. Mijangos et al., 2022). Principal Components Analysis was undertaken using the glPCA function of the R adegenet package (as implemented in dartR, Gruber et al., 2018; Jombart, 2008; Jombart & Ahmed, 2011). Principal Coordinates Analysis was undertaken using the pcoa function in R package ape (Paradis & Schliep, 2019) implemented in dartR Version 2 (J. Mijangos et al., 2022).

To exemplify the effect of missing values on SNP visualisation using PCA (Figure 7), we performed computer simulations in dartR Version 2.0.3 (J. Mijangos et al., 2022). Briefly, we simulated ten populations that reproduced over 200 non-overlapping generations. Simulated populations were placed in a linear series with low dispersal between adjacent populations (one disperser every ten generations). Each population had 100 individuals, of which 50 individuals were sampled at random. Genotypes were generated for 1,000 neutral loci on one chromosome. We then randomly selected 50% of genotypes and set them as missing data. Principal Components Analysis was undertaken using the glPCA function of the R adegenet package (as implemented in dartR, Gruber et al., 2018; Jombart, 2008; Jombart & Ahmed, 2011). R script and input files to replicate this analysis are provided in the supplementary material.

The data for the Australian Blue Mountains skink *Eulamprus leuraensis* (Figure 8) were generated for 372 individuals collected from 17 swamps isolated to varying degrees in the Blue Mountains region of New South Wales. Tail snips were collected and stored in 95% ethanol. The tissue samples were digested with proteinase K overnight and DNA was extracted using a NucleoMag 96 Tissue Kit (MachereyNagel, Duren, Germany) coupled with NucleoMag SEP (Ref. 744900) to allow automated separation of high-quality DNA on a Freedom Evo robotic liquid handler (TECAN, Miinnedorf, Switzerland). SNP data were generated by the commercial service of Diversity Arrays Technology Pty Ltd (Canberra, Australia) using published protocols (Georges et al., 2018; Kilian et al., 2012; Sansaloni et al., 2011). A total of 13,496 loci were scored which reduced to 7,935 after filtering out secondary SNPs on the same sequence tag, filtering on reproducibility (threshold 0.99) and call rate (threshold 0.95), and removal of monomorphic loci.

The algorithms discussed in this paper form the basis of the distance analysis implemented in R software package dartR (Gruber et al., 2018; J. Mijangos et al., 2022).

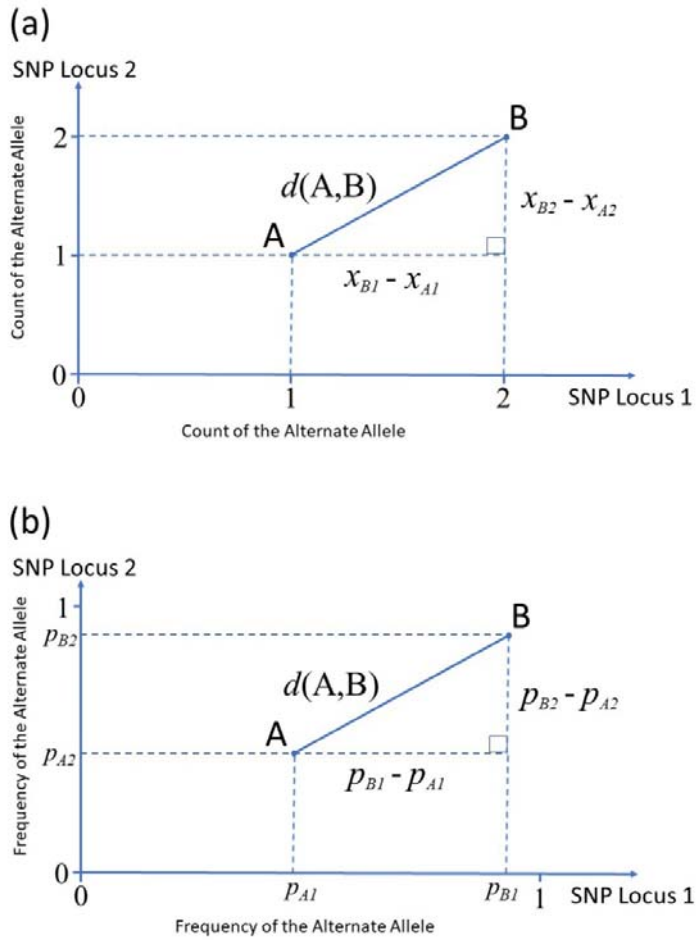

Figure 1. Distance between two points A and B represented in two-dimensional space (a) can be calculated from their Cartesian coordinates using Pythagoras' rule – the square of the hypotenuse of a right-angled triangle is equal to the sum of the squares of the two adjacent sides. Each axis can be considered to represent a locus (b), with the value taken by an individual (A or B) on that axis called as x_{Ai} and x_{Bi} = 0, 1 or 2 for SNP genotype. As such, each individual is represented by a point in a multidimensional space defined by the i = 1 to L loci. Populations A and B can be similarly depicted, as in (c), with the relative frequency of the alternate allele in the population (p_{Ai} and p_{Bi}) as the value taken on each SNP axis. Representation for SNP PA data can be similarly defined.

The Concept of Distance

Standard Euclidean distance is a common-sense notion derived as an abstraction of physical distance. Applying Standard Euclidean distance to SNP data is straightforward. Where points

A and B represent two individuals (Figure 1a), the horizontal axis represents SNP Locus 1 and the values x_{A1} and x_{B1} represent the scores for that locus (0 or 1 or 2 for SNPs; 0 or 1 for tag presence-absence) for individuals A and B respectively; the vertical axis represents SNP Locus 2 with the values x_{A2} and x_{B2} similarly defined.

$$d(A,B) = \sqrt{\sum_{i=1}^{L} (x_{Ai} - x_{Bi})^2}$$

Standard Euclidean Distance can be similarly defined for populations,

$$D(A,B) = \sqrt{\sum_{i=1}^{L} (p_{Ai} - p_{Bi})^2}$$

where p_{Ai} and p_{Bi} are the relative frequencies of the alternate allele at locus i in populations A and B respectively (Figure 1b).

The equations for *d(A,B)* and *D(A,B)* can be scaled to fall in the range [0,1] on noting that its maximum is achieved for SNP genotype data when all x_{Ai} are 0 and all x_{Bi} are 2; for SNP PA data the maximum is achieved when all x_{Ai} are 0 and all x_{Bi} are 1. Note also that the equations for *d(A,B)* and *D(A,B)* are symmetric with respect to choice of which allelic state is assigned to reference (0 for homozygous reference) and which is assigned to alternate (2 for homozygous alternate). Interchanging the reference and alternate alleles (that is applying transformation $x' = 2 - x$ for SNP genotype data or $x' = 1 - x$ for SNP PA data) has no impact on the value of the distance between A and B.

A Mantel Test (Guillot & Rousset, 2013; Mantel, 1967) can be used to compare two genetic distance matrices (or a genetic distance and a geographic distance matrix) provided they are constructed for the same entities and those entities are independent (i.e. not autocorrelated).

Generalization of the concept of Distance

Standard Euclidean distance is just one of many distance measures. The concept of distance more generally can be distilled down to three basic properties. For a metric distance we have:

$$d(A,B) = 0 \text{ if and only if } A = B$$

$$d(A,B) = d(B,A)$$

$$d(A,B) \leq d(A,C) + d(B,C)$$

The first condition asserts that indiscernible entities are one and the same. The second condition asserts symmetry. The last condition is referred to as the triangle inequality which enforces the notion that the distance between two points is the shortest path between them. From these properties we can conclude that metric distances must be non-negative.

$$d(A,B) \geq 0$$

In essence, metric distances are well-behaved distances. Standard Euclidean Distance, as with all Euclidean distances, is a metric distance.

Graphically, the metric properties make complete sense for a distance (Figure 2). Given three points defined by the distances between them, the position of each of them is defined (Figure 2a). This is necessary (though not sufficient) if we are to represent our distances in Cartesian coordinate space.

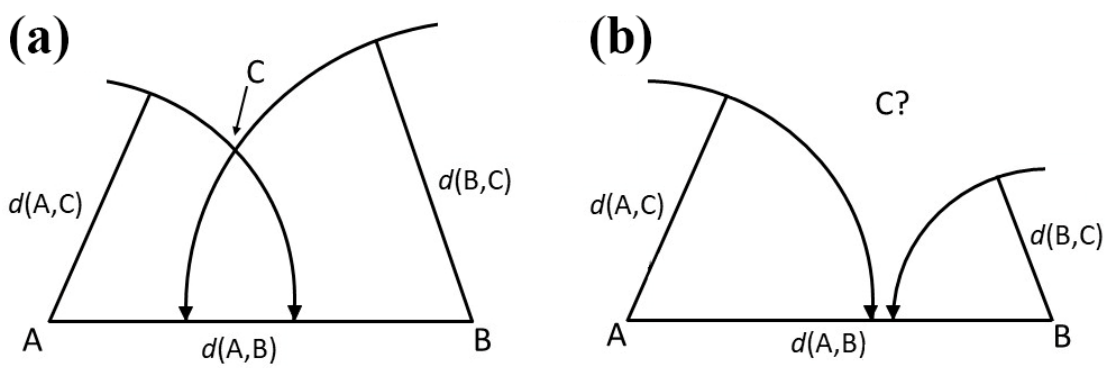

Figure 2. Visual representation of the triangle inequality as used to define a metric distance. **(a)** – if three distances between points A, B and C satisfy the metric property $d(A,B) \leq d(A,C) + d(B,C)$, then the position of each of A, B and C in space is well defined. **(b)** – if not, and $d(A,B) > d(A,C) + d(B,C)$, then point C is undefined.

While the metric properties of a distance are clearly important, many measures used in genetics are non-metric. An example of a dissimilarity measure that fails to satisfy the symmetry condition is one defined on private alleles. Private alleles are those uniquely possessed by one population when compared to other populations. The number of private alleles possessed by population A compared to population B will typically be different from the number of private alleles possessed by population B compared with A. Thus, the resultant distances will not satisfy the second metric criterion of symmetry. Other genetic distances in

use do not satisfy the triangle inequality. For example, calculating the percentage of fixed allelic differences between two entities (the percentage of alleles that are homozygous for the reference allele in one entity and homozygous for the alternate allele in the other entity) as a measure of distance satisfies the first two conditions of a metric distance, but not the triangle inequality and so is non-metric. Nor is Nei's D (Nei, 1972) a metric distance for the same reason, but the common alternative of Rogers' D (Rogers, 1972) is metric. F_{ST} is non-metric. The Bray-Curtis dissimilarity measure is non-metric but is rank-order similar to the Jaccard distance, which is metric. And so on (refer Legendre & Legendre, 2012-- tables 7.2 and 7.3).

Genetic Distances for Individuals

Binary Data

SNP PA data involves scoring SNP loci as "called" [1] or "not called" [0]. They are called because the two restriction enzymes find their mark (in DArTSeq or ddRAD), the corresponding sequence tags are amplified and sequenced, and the SNP is scored as 1 for that locus. If, however, there is a mutation at one or both of the restriction enzyme sites, then the restriction enzyme does not find its mark, the corresponding sequence tag in that individual is not amplified or sequenced, or if it is amplified from a different start site, is no longer considered homologous during SNP pre-processing, and the SNP is called as missing for that individual. The individual is scored as 0 for that locus. SNP PA data comprise dominant markers, in that both homozygotes and heterozygotes for presence of the sequence tag is scored as 1.

Taking two individuals A and B, we can count the different cases,

$$N_{00} = \sum_{i=1}^{L} \begin{matrix} 1, where\ x_{Ai} = x_{Bi} = 0 \\ 0, where\ x_{Ai} \neq x_{Bi} \end{matrix}$$

.....(1)

where x_{Ai} and x_{Bi} are the SNP PA scores for individuals A and B respectively. N_{00} is the sum of loci scored 0 (absent) for both individuals;

$$N_{10} = \sum_{i=1}^{L} \begin{matrix} 1, where\ x_{Ai} = 1 \\ 0, where\ x_{Bi} = 0 \end{matrix}$$

.....(2)

that is, sum loci scored 1 (present) for Individual A and 0 (absent) for Individual B;

$$N_{01} = \sum_{i=1}^{L} \begin{matrix} 1, where\ x_{Ai} = 0 \\ 0, where\ x_{Bi} = 1 \end{matrix}$$

.....(3)

that is, sum of loci scored 0 (absent) for Individual A and 1 (present) for Individual B;

$$N_{11} = \sum_{i=1}^{L} \begin{matrix} 1, where\ x_{Ai} = x_{Bi} = 1 \\ 0, where\ x_{Ai} \neq x_{Bi} \end{matrix}$$

.....(4)

that is, sum of loci scored 1 (present) for both individuals. These summations do not include loci for which data are missing (NA) for one or both individuals.

The number of loci L is given by

$$L = N_{00} + N_{01} + N_{10} + N_{11}$$

.....(5)

Based on the above intermediates [(1) – (5)], there are several ways to calculate a binary distance between two individuals (Choi et al., 2010), some of which are shown in Table 1. The two most commonly used distance measures are Standard Euclidean Distance and Simple Matching Distance (Sokal & Michener, 1958). Both are based on the number of mismatches between two individuals ($N_{01} + N_{10}$), expressed as a proportion of the total number of loci considered (L), and used when there is symmetry (equivalence) in the information carried by 0 (absence) and 1 (presence). Simple Matching Distance is simply the Standard Euclidean distance squared. It is a metric distance.

The Jaccard Distance is a variation on the Simple Matching Distance that down-weights joint absences and so is no longer symmetric with respect to 0 and 1 scores. Down-weighting absences of the sequence tags is arguably what you do not want for data comprised of counts of sequence tag absences arising from a positive event, that of a mutation at one (or both) of the restriction enzyme sites. If you wish to use the Jaccard Distance on DArT or ddRAD SNP PA data, you might consider recoding the data ($x' = 1 - x$) so that 1 represents presence of a mutation at one or both of the restriction enzyme sites (i.e. absence of the amplified tag) and 0 represents absence of such a disruptive mutation (i.e. success in amplifying the sequence tag). Having made this simple adjustment, the Jaccard Distance will

down-weight joint absence of a disruptive mutation. The Jaccard Distance is a metric distance (Levandowsky & Winter, 1971).

Table 1. Some genetic distances commonly applied to binary SNP data, that is, to SNP PA data. Variables are described in the text. Formulae are presented to illustrate the adjustments made to the denominator for Jaccard and Sørensen distances rather than their conventional algebraic form.

Name	Formula	Alternate Names	Reference
Standard Euclidean Distance	$d_E = \sqrt{\frac{N_{01} + N_{10}}{L}}$		
Simple Matching Distance	$d_{SM} = \frac{N_{01} + N_{10}}{L}$	Scaled Hamming Distance	(Sokal & Michener, 1958)
Jaccard Distance	$d_J = \frac{(N_{01} + N_{10})}{L - N_{00}}$	Marczewski-Steinhaus D (Marczewski & Steinhaus, 1959); Ružička D; Soergel D	(Jaccard, 1912)
Sørensen Distance	$d_S = \frac{(N_{01} + N_{10})}{L - N_{00} + N_{11}}$	Dice D (Dice, 1945)	Sørensen D (Sørensen, 1948)

The Sørensen Distance adjusts the denominator to down-weight the joint absences (0,0) and up-weight joint presences (1,1). As with the Jaccard Distance, you might consider reversing the scores for absence (0) and presence (1) to 1 and 0 respectively when dealing with DArT or ddRAD SNP PA data. Special attention may be required to manage missing values when applying the Sørensen and Jaccard Distances, because adjustment of the denominator in their formulae (Table 1) can lead to a potential systematic bias (Orlóci, 1978:62). The Sørensen Distance is not a metric distance (Orlóci, 1978:61).

Software packages in R for calculating distances for binary data, including those listed above using the same terminology, include vegan (Oksanen et al., 2014) and dartR (Gruber et al., 2018; J. Mijangos et al., 2022).

SNP Genotype Data

Unlike binary data, SNP data take on three values at a locus: 0 for homozygous reference allele; 1for heterozygous; and 2 for homozygous alternate allele. This scoring scheme is convenient computationally because the value adopted represents the count of the alternate allele. Software packages PLINK (Chang et al., 2015), SNPTEST (Marchini et al., 2007) and dartR (Gruber et al., 2018; J. Mijangos et al., 2022) use this scoring scheme as the default. The alternative scoring scheme of 0 for homozygous reference allele, 1 for homozygous alternate allele and 2 for heterozygotes is commonly used in genome-wide association studies (GWAS) and software such as GCTA (Genome-wide Complex Trait Analysis, Yang et al., 2011) and by Diversity Arrays Technology Pty Ltd (Canberra, Australia).

Standard Euclidean Distance as applied to SNP genotype data is defined in the usual way with loci as the axes in a coordinate space, and the value on each axis is 0, 1 or 2 as defined above. The scaling factor of ½ arises because the maximum squared distance between two individuals at a locus is $(2\text{-}0)^2 = 4$.

$$d_E(A,B) = \frac{1}{2}\sqrt{\sum_{i=1}^{L}\frac{(x_{Ai}-x_{Bi})^2}{L}}$$

where x_{Ai} and x_{Bi} are the counts of the alternate allele at locus i for individual A and B respectively; L is the number of loci for which both x_{Ai} and x_{Bi} are non-missing.

The Simple Matching Distance uses the counts of shared alleles between two individuals i and j at a locus is given by

$$\begin{aligned} c_{i,j} &= 0, where\ no\ alleles\ are\ shared\ [0,2]\ or [2,0] \\ &= 1, where\ one\ allele\ is\ shared\ [0,1]\ or\ [2,1]\ or\ [1,2] \\ &= 2, where\ both\ alleles\ are\ shared\ [0,0]\ or\ [1,1]\ or\ [2,2] \end{aligned}$$

and is calculated as

$$d_{SM}(A,B) = 1 - \frac{1}{2L}\sum_{i=1}^{L} c_{ij}$$

where L is the number of loci non-missing for both individuals i and j. It is metric and similar to the Allele Sharing Distance (Gao & Starmer, 2007), differing from it by a factor of 2.

Czekanowski Distance (Czekanowski, 1913) is calculated by summing the scores across the axes

$$d_{Cz}(A,B) = \frac{1}{2L}\sum_{i=1}^{L}|x_{Ai} - x_{Bi}|$$

where x_{Ai} and x_{Bi} are the counts of the alternate allele at locus i for individual A and B respectively; L is again the number of loci for which both x_{Ai} and x_{Bi} are non-missing. Referred to also as the Manhattan D or the City Block D, Czekanowski Distance is a metric distance.

Software packages in R for calculating distances for SNP genotype data, including those listed above using the same terminology, include vegan (Oksanen et al., 2014), adegenet (Jombart, 2008), poppr (Kamvar et al., 2014), pegas (Paradis, 2010) and dartR (Gruber et al., 2018; J. Mijangos et al., 2022).

SNP genotype data can be converted to binary data by counting the shared alleles between two individuals i and j at a locus

$$c_{i,j} = 0, no\ alleles\ are\ shared\ [0,2]$$
$$= 1, one\ or\ both\ alleles\ are\ shared\ [0,0] \text{ or } [0,1] \text{ or } [1,2] \text{ or } [2,2]$$

whereby distance measures devised for binary data can be applied. These distances are in effect considering only absolute allelic differences between the two individuals.

Genetic Distances for Populations

Standard Euclidean Distance as applied to allele frequencies is defined in the usual way with loci (attributes) represented by the axes in a coordinate space, and the values (states) for each population (entity) on each axis set to the frequency of the alternate allele for the respective locus (Table 2). An appropriate scaling factor is applied to bring the value of the distance into the range [0,1], which renders it identical to Roger's D (Rogers, 1972). Standard Euclidean distance is a model-free metric distance, in that its formulation makes no assumptions regarding evolutionary processes generating the genetic distances.

Nei's standard genetic distance (Nei, 1972) is favoured by some over Standard Euclidean Distance because of its relationship to divergence time. When populations are in mutation-drift balance throughout the evolutionary process and all mutations result in new alleles in accordance with the infinite-allele model, Nei's *D* is expected to increase in proportion to the time after divergence between two populations (Nei, 1972). Nei's *D* is non-metric.

Reynolds genetic distance (Reynolds et al., 1983) is also approximately linearly related to divergence time in theory, but unlike Nei's Standard Genetic Distance, it is based solely on a drift model and does not incorporate mutation. Thus, it may be more appropriate than Nei's distance for spatial population genetics (divergence on relatively short timescales) and in particular, representation of genetic similarity in trees (phenograms) or networks where branch lengths need to be interpretable. A better approximation (Reynolds et al., 1983) of the linear relationship with time is given by

$$D'_{Reynolds}(A,B) = -ln[1 - D_{Reynolds}(A,B)]$$

Reynold's *D* is non-metric.

Chord Distance (A. W. F. Edwards & Cavalli-Sforza, 1964) (Table 2) assumes divergence between populations is via drift alone, an so again may be more appropriate than Nei's *D* for spatial population genetics. Chord Distance is based on Geodesic or Angular Distance (Bhattacharyya, 1946; A. W. F. Edwards, 1971; A. W. F. Edwards & Cavalli-Sforza, 1967),

$$cos\ \alpha = \sqrt{p_{Ai}p_{Bi}} + \sqrt{q_{Ai}q_{Bi}}$$

where α is the Angular Distance. For a geometric interpretation using biallelic data, see Nei & Kumar (2000:267). Chord Distance approximates Angular Distance by replacing the arc distance with the length of the corresponding straight-line segment (A. W. F. Edwards & Cavalli-Sforza, 1964) (Table 2). It is a metric distance that can be transformed to be approximately Euclidean (A. W. F. Edwards, 1971). As with Reynold's *D*, Chord Distance is proportional to shallow divergence time (for relatively small values of D) under specified assumptions (A. W. F. Edwards, 1971). It may be preferred over the model-free Standard Euclidean Distance where an underlying genetic model of divergence with time is preferred.

Bray-Curtis Distance applies to abundances and down-weights joint absences in a manner analogous to the Jaccard Distance. There is some confusion arising from the translation of Bray-Curtis Distance (Bray & Curtis, 1957) from an ecological perspective to a genetics perspective. When applied to SNP data, the Bray-Curtis Distance uses the abundance of the alternate allele which renders it asymmetric with respect to the arbitrary choice of which is reference and which is alternate allele. Bray-Curtis Distance defined in this way should thus not be used on SNP data. Some authors have suggested that the Bray-Curtis equation be applied separately to the counts of each allele, averaged for the locus, for

computing genetic distance between individuals or populations (Allele Frequency Difference or AFD, Berner, 2009) (Bray-Curtis/Allele Frequency Difference or BCAFD, Sherwin, 2022). However, when this formulation is applied to either individuals or populations, it becomes algebraically equivalent to the Czekanowski Distance (=Manhattan D). It no longer has the properties of Bray-Curtis Distance (having been rendered metric and symmetric). To avoid confusion, members of this class of distances should not be referred to as Bray-Curtis, AFD or BCAFD, but rather as Czekanowski Distance or the more familiar Manhattan D, given that naming of a distance measure should not be context dependent. The same issue has arisen in ecology (Yoshioka, 2008). Czekanowski Distance applied to SNP data and corrected for maximum value dependency, referred to as ^{A}A Distance (Sherwin, 2022), restores the asymmetry with respect to choice of reference and alternate allele.

Table 2. Some genetic distances commonly applied to populations . p_{Ai} is the proportion of the alternate allele for Locus i in population A, p_{Bi} is the proportion of the alternate allele for Locus i in population B. q_{Ai} and q_{Bi} are similarly defined for the reference alleles. L is the number of called loci.

Name	Formula	Reference
Standard Euclidean Distance	***Binary P/A*** $$D_E(A,B) = \sqrt{\frac{1}{L}\sum_{i=1}^{L}(p_{Bi} - p_{Ai})^2}$$ ***SNP genotypes*** $$D_E(A,B) = \frac{1}{2}\sqrt{\frac{1}{L}\sum_{i=1}^{L}(p_{Bi} - p_{Ai})^2}$$	
Czekanowscki Distance (Manhattan D)	$$D_{cz}(A,B) = \frac{1}{2L}\sum_{i=1}^{L}(\lvert p_{Bi} - p_{Ai})\rvert$$	(Berner, 2009, as AFD; Czekanowski, 1913)
Nei Standard Genetic Distance	$$D_{Nei}(A,B) = -ln\left(\frac{\sum_{i=1}^{L}([p_{Ai}p_{Bi} + q_{Ai}q_{Bi}]}{\sqrt{\sum_{i=1}^{L}[(p_{Ai}^2 + q_{Ai}^2]}\sqrt{\sum_{i=1}^{L}[(p_{Bi}^2 + q_{Bi}^2]}}\right)$$	(Nei, 1972)

Reynolds Distance	$D_{Reynolds}(A,B) = \sqrt{\frac{\sum_{i=1}^{L}[(p_{Ai}-p_{Bi})^2+(q_{Ai}-q_{Bi})^2]}{2\sum_{i=1}^{L}(1-p_{Ai}p_{Bi}-q_{Ai}q_{Bi})}}$	(Reynolds et al., 1983)
Chord Distance	$D_{Chord}(A,B) = \sqrt{1-\frac{1}{L}\sum_{i=1}^{L}\sqrt{p_{Ai}p_{Bi}}+\sqrt{q_{Ai}q_{Bi}}}$	(A. W. F. Edwards & Cavalli-Sforza, 1964)
Wright's paired F_{ST}	Paired F_{ST} requires estimation with approaches such as that used by R package *hierfstat* [genet.dist(gl, method="WC84")]	(Wright, 1951)

Wright's *F-statistics* (Wright, 1951) describe the distribution of genetic diversity within and between populations (Holsinger & Weir, 2009). *F-statistics* are defined in terms of the proportion of heterozygotes observed (H_{obs}) and the proportion of heterozygotes expected under Hardy-Weinberg equilibrium (H_{exp}), as follows:

$$F = 1 - \frac{H_{obs}}{H_{exp}}$$

A deficit in the observed proportion of heterozygotes compared with that expected under Hardy-Weinberg equilibrium will yield a Wright's *F* that deviates from zero. If, at a single locus, observed and expected heterozygosity are in agreement, then $F=0$. If, at the extreme, no heterozygotes are observed, then $F=1$. If there is an excess of heterozygotes, F will be less than zero. When *F* is averaged across a large number of independent loci for a population, *F* will typically fall between zero and one. If two populations that differ in allelic profiles are combined (pooled), then Hardy-Weinberg equilibrium is not a sensible null expectation. Divergence of the two allele frequency profiles will manifest as non-random mating and a departure from Hardy-Weinberg equilibrium (the "Wahlund Effect", Wahlund, 1928). Wright's *F* applied to the pooled populations is thus informative when examining population subdivision, because the resultant deficit in heterozygotes is an indication of genetic structure. *F* applied in this way incorporates two components -- one arising from departure from Hardy-Weinberg expectation within each population and the second arising from departure from Hardy-Weinberg expectation because of structure between the two populations or sub-populations. For this reason, Wright's pairwise F_{ST} is most commonly used as a measure of genetic distance between two populations or sub-populations (see Nei,

1977; Nei & Kumar, 2000:236). F_{ST} *is* a measure of the heterozygosity attributable to differences in allelic frequency profiles between populations or sub-populations, having partitioned out the contributions from departure from Hardy-Weinberg expectation within populations or sub-populations. Although not a genetic distance in the strict sense, F_{ST} can be viewed a non-metric distance that varies in value between 0 and 1, such as in studies of isolation by distance. Several methods to estimate F_{ST} have been developed and some are complex (Excoffier, 2001; Weir & Cockerham, 1984), but there are many software packages available to estimate F_{ST} for SNP genotype data (e.g. R package hierfstat, Goudet, 2005).

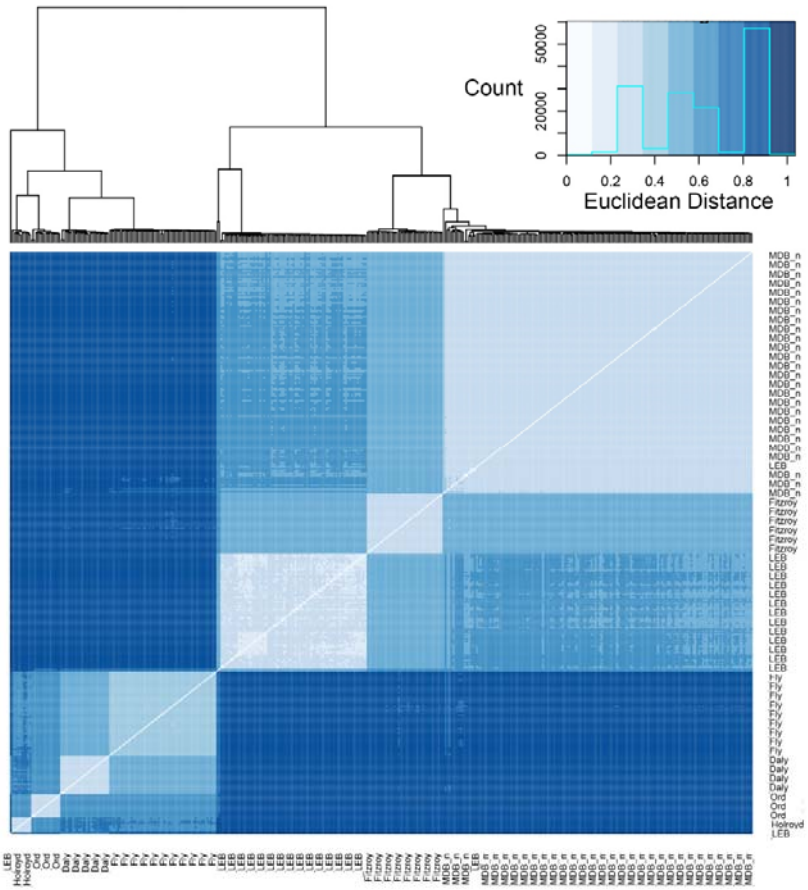

Figure 3. A heatmap used to graphically represent Standard Euclidean Distances calculated pairwise for individual in the freshwater turtle SNP dataset, labelled by sampling locality. Individuals are ranked to highlight clusters. Not all individuals are shown. The legend gives the colour scale used to depict the distances with white for zero distance (hence the white diagonal) and darkest blue for the maximum distance of one. The heatmap is accompanied by a neighbour-joining tree. The existence of strong structure is immediately evident.

Software packages in R for calculating distances for binary data, including those listed above using the same terminology, include vegan (Oksanen et al., 2014), adegenet (Jombart, 2008), poppr (Kamvar et al., 2014), pegas (Paradis, 2010) and dartR (Gruber et al., 2018; J. Mijangos et al., 2022). Package heirfstat (Goudet, 2005) is the software of choice for calculating F statistics.

Finally, SNP genotype data at the population level can be converted to binary data by counting the shared alleles between two populations A and B at a locus

$$c_{A,B} = 0, no\ alleles\ are\ shared\ by\ the\ two\ populations\ at\ that\ locus$$
$$= 1, one\ or\ both\ alleles\ are\ shared\ at\ that\ locus$$

whereby distance measures devised for binary data can be applied. These distances are, in effect, considering only fixed allelic differences between the two populations.

Visualization of SNP data

There are several methods for visualizing a distance matrix. Perhaps the simplest is to represent the distance matrix as a heat map where colour is used to signify the magnitude of distance between pairs of individuals (Figure 3). Note the white diagonal which indicates zero differences between individuals and themselves, and that the heatmap is redundant in the sense that the lower and upper triangles are identical. A high degree of population structuring is immediately evident. For example, individuals of *Emydura* from the north basin of the Murray-Darling catchment are very similar to each other genetically, but very different from populations of *Emydura* from northern Australia and New Guinea. Heatmaps are a very quick way of gaining a visual indication of the level of structuring across the dataset.

The heatmap is accompanied by a neighbour-joining tree (Saitou & Nei, 1987) which is another common way of graphically representing genetic similarity among entities (Figure 3), not to be confused with their phylogenetic application. Note that to be faithfully represented in tree form, that is, without distortion, the distance measure must be both metric and satisfy the four-point condition (for any four individuals, a branching structure linking them has an internal branch ≥ 0) (Buneman, 1974). This is rarely the case. An alternative clustering algorithm is UGPMA (unweighted pair group method with arithmetic mean, Sokal & Michener, 1958).

By far the most common approach to visualizing genetic distance analysis is Principal Components Analysis (Hotelling, 1933; Jolliffe, 2002; Pearson, 1901) or PCA. PCA takes a

549 SNP data matrix (SNP genotypes or SNP PA data), represents the entities (individuals or
550 samples or specimens) in a space defined by the *L* loci, centres and realigns that space by
551 linear transformation (rotation) to yield new orthogonal axes ordered on the contribution of
552 variance (represented by their eigenvalues) in their direction (defined by their eigenvector).
553 This process maintains the relative positions of the entities. Because the resultant axes are
554 ordered on the amount of information they contain, the first few axes, preferably the first two
555 or three, tend to contain information on any structure in the data (signal) and later axes tend
556 to contain only noise (Gauch, 1982). This is a powerful visual technique for examining
557 structure in the SNP dataset. An example of a PCA is presented in Figure 4. Important
558 variations include the combination of PCA with discriminant analysis (DAPC, Jombart et al.,
559 2010), adjustment for confounding factors (AC-PCA, Lin et al., 2016) and accommodating
560 sample allelic dropout from low read depth sequence tags (PCAngsd, Meisner & Albrechtsen,
561 2018). The potential for sNMF (Sparse Non-negative Matrix Factorization) a PCA-related
562 technique useful for disentangling signal from different dyes in microscopy (Rabinovich et
563 al., 2003), may have application in population genetics, particularly for identifying cases of
564 admixture (Frichot et al., 2013). For a broader discussion on variations of PCA not restricted
565 to SNP data, refer to Abegaz et al. (2019). Specific R software for undertaking classical PCA
566 on SNP data and displaying the results graphically includes adegenet (glPCA, Jombart, 2008)
567 and the implementation of adegenet::glPCA in dartR (Gruber et al., 2018; J. Mijangos et al.,
568 2022), though there is a wide range of packages for undertaking and displaying this very
569 popular analysis.

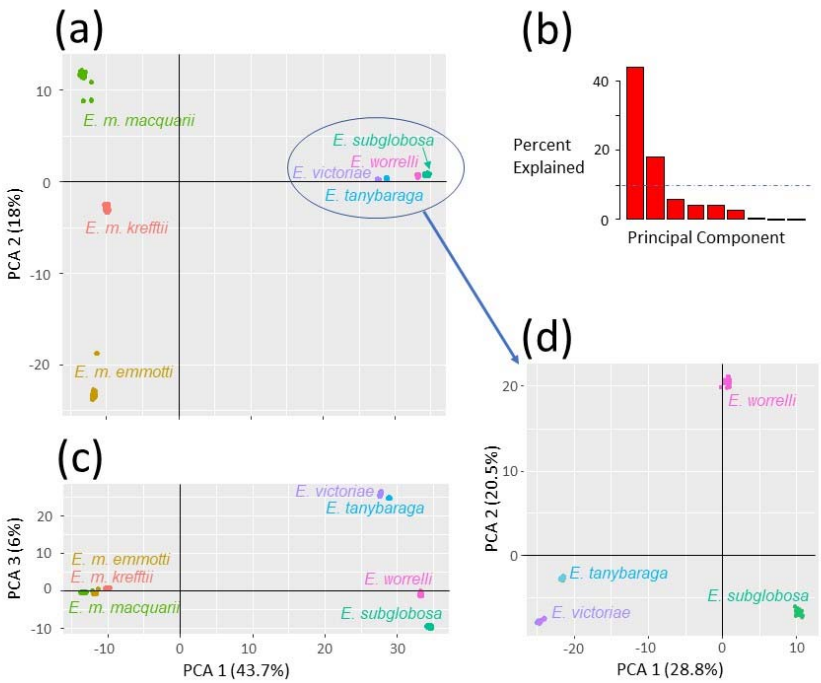

Figure 4. Principal Components Analysis (PCA) as applied to a SNP genotype dataset for the Australasian species of *Emydura* (Chelonia: Chelidae). Plot of individuals in a two-dimensional space (**a**) defined by Principal Component 1 (horizontal axis) and Principal Component 2 (vertical axis). Together they explain 61.7% of the total variance among individuals. A scree plot (**b**) shows the contributions to variance by the first nine principal components (those that exceed the Kaiser-Guttman threshold, Guttman, 1954) of which only two each explain more than 10% of variation. Proximity of *Emydura subglobosa/worrelli* to *E. victoriae/tanybaraga* in 2D obscures their distinction, evident when Principal Component 3 is considered (**c**). The variation explained by the first two principal components can be largely set aside by considering structure within the cluster *Emydura subglobosa/worrelli/victoriae/tanybaraga* only (**d**), effectively allowing consideration of deeper dimensions.

Had the data been drawn from a panmictic population (arguably the null proposition), each of the original variables would, on average, be expected to capture the same quantity of variance, and the ordination would fail (the first two axes would each represent only a small and random percentage of the total variance). When there is structure, the percentage of variation explained by the top principal components will dominate the ordination. However, the absolute value of the percentage variation explained cannot be compared across studies as a measure of the strength of the result as if it is some sort of absolute; the percentage variance explained by a principal component needs to be taken in the context of the average percent variation explained by all components (Guttman, 1954). Variances explained by the top 2 or 3 principal components in SNP analyses are typically quite low, but this does not necessarily

592 indicate a poor outcome, as these values need to be considered in the context of the number
593 of axes in the overall ordination.

594 Note also that a PCA plot can be misleading if one chooses, for convenience, only two or
595 three dimensions in which to visualize the solution. For example, separation in a 2-D plot can
596 be accepted as real but proximity cannot because further separation can occur in deeper
597 dimensions each coupled with a substantial explained variance (Figure 4c). A widely used
598 approach to determining the number of dimensions for the final solution is to examine a scree
599 plot (Cattell, 1966), where the eigenvalues (proportional to variance explained) associated
600 with each of the new ordered dimensions are plotted (Figure 4b). It is usual to apply the
601 Kaiser-Guttman criterion (Guttman, 1954) whereby only those dimensions with more than
602 the average eigenvalue are considered, or to apply a related but less conservative approach to
603 take into account sampling variability (Jolliffe, 1972). Even so, this may result in many
604 informative dimensions. One must decide how much information to discard (say, for
605 example, keeping only those components that explain more than 10% of total variation) or
606 adopt, as a threshold, a visually evident sudden drop in the percentage variation explained,
607 commonly referred to as an elbow. More formal techniques (Cangelosi & Goriely, 2007;
608 Jackson, 1993; Peres-Neto et al., 2005) include the broken-stick approach of Macarthur
609 (1957), which provides a good combination of simplicity of calculation and evaluation of
610 suitable dimensionality (Jackson, 1993). The broken-stick model retains components that
611 explain more variance than would be expected by randomly dividing the variance into equal
612 parts. Another related approach is to observe that the eigenvalues of lower "noise"
613 dimensions are likely to decline geometrically, a trend that can be linearized by a log
614 transformation. Informative dimensions are those for which variance explained greatly
615 exceeds that predicted by this linear trend line (more strictly, exert disproportionate leverage
616 on the regression). A more recent approach assesses the statistical significance of the
617 variation explained by each Principal Component (Patterson et al., 2006). Under specified
618 assumptions, the sampling distribution of the ordered eigenvalues, under the null hypothesis
619 of no structure in the data, follows Tracy-Widom distribution (Tracy & Widom, 1993). Thus,
620 it is possible to assign a probability to an observed eigenvalue and retain for consideration
621 only those principal components that are statistically significant (Patterson et al., 2006). The
622 technique is sufficiently robust to violations of its underlying assumptions (e.g. normality) to
623 be applicable to large genetic biallelic data arrays.

Displaying the results in a two-dimensional plot is straightforward. Various software packages can display the results in three dimensions and allow rotation of the three axes to provide the best perspective (e.g. dartR, Gruber et al., 2018; J. Mijangos et al., 2022). Higher dimensions can be visualized by plotting the set of largest components in pairwise fashion (Figure 4a,c). Alternatively, if there are strong groupings in the PCA plot in two dimensions, individuals in each of these groupings can be isolated and analysed by PCA separately (Figure 4d) (Georges & Adams, 1992).

Generalization of PCA

Principal Co-ordinates Analysis (PCoA, Gower, 1966) is an alternative visualization technique that represents a distance matrix in a Euclidean space defined by an ordered set of orthogonal axes, as does PCA. Again, the axes are ordered on the amount of information they contain so that the first few axes tend to contain information on any structure in the data (signal) and later axes tend to contain only noise (Gauch, 1982). Important variations include adjustment for confounding factors (AC-PCoA, Wang et al., 2022) and application of iterative procedures to best match measured distances against distances in the visual representation for a specified number of dimensions (Belbin, 1991; Kruskal, 1964; Shepard, 1962; Torgerson, 1952). Note that the adjective 'metric' here is a reference to measurement of a continuous variable, not in the geometric sense applied elsewhere in this paper. Perhaps a better alternative to non-metric MDS would be rank-order MDS. R Software for generating the input distance matrix, then undertaking and displaying graphically a PCoA is provided by ape (function pcoa, Paradis & Schliep, 2019) and the implementation of ape::pcoa in dartR (Gruber et al., 2018; J. Mijangos et al., 2022).

The primary difference between PCA and PCoA is that PCA works with the covariance (or correlation) matrix derived from the original data whereas PCoA works with any distance matrix. Because the mathematics of PCA moves forward from the covariance (or correlation) matrix, the insight attributed to John Gower (1966) was to substitute any distance matrix for the covariance (or correlation) matrix at this point in the analysis, following a simple transformation. This yields an ordered representation of those distances, metric or otherwise, in multivariable space akin to PCA, greatly expanding the range of application of ordination. In PCA, the N individuals are represented in a space of L dimensions defined by the loci whereas in PCoA, the individuals are represented in an N-1

space with coordinate axes based solely on their pairwise distances. Thus, the PCoA is not implicitly connected to any raw SNP genotype or SNP PA data.

Choosing the number of dimensions to display in visualizing a PCoA is similar to PCA. Missing values are less disruptive for PCoA than classical PCA because they are accommodated in pairwise fashion rather than globally, but they nevertheless require consideration. Missing values result in variation in the precision of estimates of allele frequencies across loci and can break the Euclidean properties of a sample distance matrix even when the chosen metric is Euclidean in theory. Other considerations arise in PCoA from using distance matrices that are non-Euclidean. If a Euclidean distance matrix is used in PCoA then, in the absence of missing values, the distances in the input matrix are represented faithfully in the full ordinated space, that is, without distortion (Figure 5c). The same cannot be said of metric distances in general, as the metric properties ensure that the individuals can be represented in an ordinated space, but not in a rigid linear space (Figure 5a, after Gower, 1982) – some curvature of the space may be required to satisfy the triangle inequality (Figure 5b), which will potentially compromise ability to faithfully represent the distances between entities in a space defined by Cartesian coordinates.

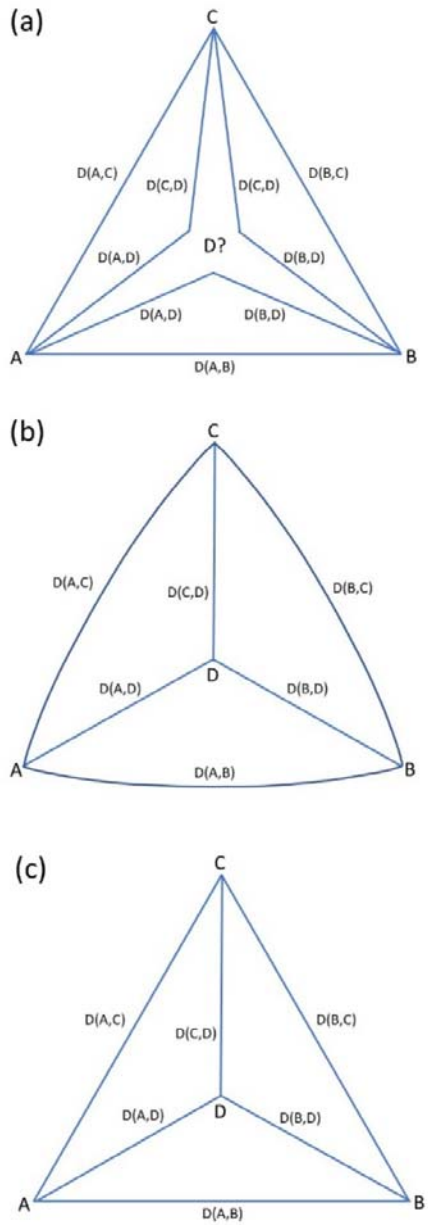

Figure 5. Metricity is not sufficient to represent distances in a rigid space defined by Cartesian coordinates; the distances must be Euclidean. **(a)** – distances between four individuals that satisfy the metric properties can nevertheless not be represented in a linear space defined by Cartesian coordinates because the position of individual D is not defined by the interindividual distances (after Gower, 1982). **(b)** – this distortion can be resolved by allowing non-linear links (geodesics) to represent distances between individuals as, in the case of four points shown, the edges of an irregular Reuleaux tetrahedron in three dimensions. **(c)** – in contrast, Euclidean distances between four individuals can always be represented by linear segments without distortion, as edges of an irregular conventional tetrahedron in three dimensions.

Distortion arising from using non-Euclidean distances manifests as displacement of the points in the visualization, so that the distances among them no longer fundamentally represent the input values; some eigenvalues will be negative (representing imaginary eigenvectors) (Gower & Legendre, 1986). However, the level of distortion is likely to be of concern only if the absolute magnitude of the largest negative eigenvalue is greater than that of any of the dimensions chosen for the reduced representation (Cailliez & Pages, 1976). A few small negative eigenvalues do not detract much from the visual representation if only a few of the highest dimensions are retained in the final solution (Sibson, 1979). Thus, departure from theory needs to be addressed in practice only if it causes serious issues. Note also that a distance measure does not need to be metric or Euclidean in theory for a sample distance matrix itself to be metric or Euclidean.

Negative eigenvalues complicate interpretation of the variance contributions. In particular, one can no longer calculate the percentage variation explained by a PCoA axis by expressing the value of its eigenvalue over the total sum of the eigenvalues. A correction is necessary (Legendre & Legendre, 2012:506).

$$\%\ explained\ = \frac{e_i + k}{\sum_{i=1}^{N} e_i + (N-1)k}$$

where e_i is the eigenvalue for PCoA axis i, N is the number of entities, and k is the absolute magnitude of the largest negative eigenvalue.

If negative eigenvalues are considered problematic for the reduced space representation, a transformation can render them all positive and the distance matrix Euclidean. Common transformations put to this purpose are the square root (Legendre & Legendre, 2012:501), Cailliez transformation (Cailliez, 1983; Gower & Legendre, 1986) and the Lingoes transformation (Gower & Legendre, 1986; Lingoes, 1971).

Square root $\quad \mathrm{D}'(\mathrm{A},\mathrm{B}) = \sqrt{\mathrm{D}(\mathrm{A},\mathrm{B})}$

Cailliez $\quad \mathrm{D}'(\mathrm{A},\mathrm{B}) = \mathrm{D}(\mathrm{A},\mathrm{B}) + \mathrm{c} \quad$ for all $A \neq B$

Lingoes $\quad \mathrm{D}'(\mathrm{A},\mathrm{B}) = \sqrt{\mathrm{D}(\mathrm{A},\mathrm{B}) + \mathrm{c}} \quad$ for all $A \neq B$

The value of c is chosen to be the smallest value required to convert the most extreme negative eigenvalue to zero.

711 Finally, the outcome of a PCoA with an input matrix comprised of Standard
712 Euclidean Distances calculated from individual genotypes is identical to the outcome of a
713 PCA (Cox & Cox, 2001:43-44). In this context, the interchangeability of the two, PCA and
714 PCoA leads to confusion on the distinction between the two analyses. Notwithstanding that
715 the final arrangement of entities in the ordinated space is the same for PCA and PCoA using
716 Euclidean Distance on the same data, the percentage variation explained by each axis will
717 differ between the two approaches. This is because PCA calculates the percentage variation
718 explained by an axis as the percentage its eigenvalue represents of the total of all eigenvalues.
719 The eigenvalues are calculated from the covariances generated from the input data matrix.
720 PCoA in contrast will calculate the percentage variation explained by an axis as the sums of
721 squares of the distance between the entities in the direction of the axis expressed as a
722 percentage of the total sums of squares of the distances in the input distance matrix. The two
723 will differ even when PCA and PCoA using Euclidean Distance are applied to the same data.
724 Hence, it is not possible to simply compare the performance of a PCA and a PCoA based on
725 examination of the values of percentage variation explained.

726 **Missing values**

727 SNP datasets typically have substantial numbers of missing values. With SNP genotypes,
728 missing data can arise because the read depth is insufficient to detect SNP-containing
729 sequence tags consistently, or because mutations at one or both of the restriction enzyme
730 recognition sites in some individuals result in allelic drop-out (null alleles). Because those
731 that arise from mutation are inherited, they are subject to genetic drift (and potentially local
732 selection) differentially within each sampled population, and so are not randomly distributed
733 across the entire dataset. Indeed, in some populations the mutation(s) leading to missing
734 values may become fixed. Filtering on call rate with a threshold applied globally will
735 potentially admit high frequencies of missing data at particular loci for single populations,
736 which has consequences as outlined below. In SNP PA data, the null alleles are themselves
737 the data, scored as presence or absence of the amplified tag. In this context, missing values
738 arise because the read depth is insufficient to be definitive about the absence of a particular
739 sequence tag.

740 Missing data are problematic for distance analyses. Techniques like classical PCA
741 that access the raw data matrix cannot accommodate missing data. PCoA, which accesses a
742 distance matrix, is less affected by missing values in the raw data (Rohlf, 1972) because they
743 are eliminated in pair-wise fashion during construction of the distance matrix, not globally.

PCoA is nevertheless affected by missing values in ways that are not immediately transparent – by potential breakage of metric or Euclidean properties, and by disregarding variable precision of estimates of population allele frequencies across loci. The trade-off is one of balancing data loss with bringing distortion of the visual representation within acceptable levels.

Because classical PCA will not accept missing values, when a locus is not scored for a particular individual, either the data for the entire locus must be deleted or the data for the entire individual must be deleted. This is clearly very wasteful of information, and unacceptable loss when working with SNP datasets. The data loss can be controlled to an extent by pre-filtering on call rate by locus (say requiring a call rate > 95%) or by individual (say requiring a call rate > 80%), but the remaining missing data may still need to be managed. Numerous ways for handling missing data in PCA have been suggested (Dray & Josse, 2015), but the most common method is to replace a missing value at a locus with the mean of the allele frequencies for that locus calculated across the individuals for which it is non-missing (mean-imputed missing data).

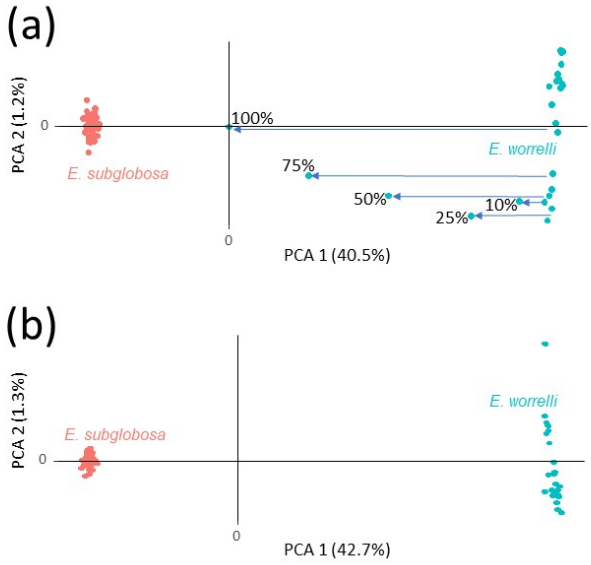

Figure 6. Principal Components Analysis (PCA) applied to two populations where five individuals in one population (*Emydura worrelli*) have had percentages of SNP loci ranging from 10% to 100% set to missing. **(a)** – the impact of this on PCA where missing data are filled with the average global allele frequencies (mean-imputed missing data) is clear, and subject to misinterpretation as hybridization or

various levels of introgression. **(b)** – the issue can be resolved by local imputation, in this case by nearest-neighbour imputation.

Mean-imputation of missing data can lead to the individuals with a high proportion of missing data being drawn out of their natural grouping and toward the origin, leading to potential misinterpretation (Yi & Latch, 2021). For example, if individuals in the PCA aggregate into natural clusters, perhaps representing geographic isolates, and these clusters are on either side of the origin, then an individual with a high frequency of missing values will be drawn out of its cluster when the missing values are replaced by the global average allele frequencies (Figure 6). Its location intermediate to the two clusters might be falsely interpreted as a case of admixture. Individuals with missing data corrected by mean-imputation will also distort confidence envelopes as applied to clusters with consequences for interpretation (Figure 7).

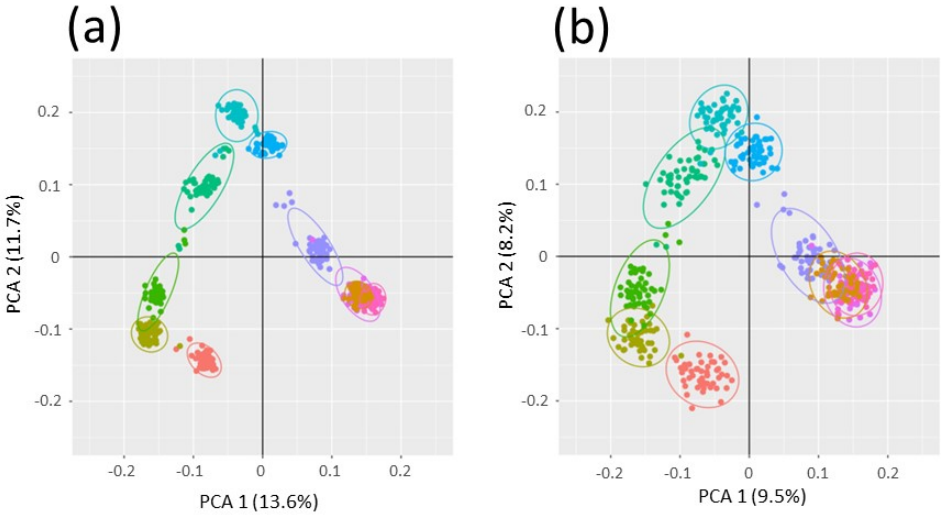

Figure 7. A PCA plot of simulated data showing aggregations and their associated 95% confidence limits for (a) data with no missing values and (b) data with 50% missing values. Note the inflationary effect missing values have on the spread of the points in each aggregation, manifested as inflation of the 95% confidence limits, an artifact. This is of particular relevance to studies of population assignment.

Better ways than global mean-imputation for handling missing values in SNP datasets include local filtering for call rate or local imputation. There are several options:

1. Apply a threshold for an acceptable call rate separately to each population, noting that a missing value rate of <10% causes only modest distortion (Figure 6a). Filtering on call

788 rate by population with a threshold of 95% can be expected to constrain the distortion
789 satisfactorily.

790 2. Replace missing values for an individual with the mean observed allele frequency for the
791 population from which the individual was drawn. In this way, the individual is displaced
792 toward the centroid of the population from which it was drawn, not the origin of the
793 PCA.

794 3. Replace missing values for an individual with the expected value for the population to
795 which the individual belongs, based on the assumption of Hardy-Weinberg equilibrium.
796 The individual will again be displaced toward the centroid of the population from which
797 it was drawn, not the origin of the PCA.

798 4. Replace missing values for an individual by the value at the same locus from its nearest
799 neighbour (the individual closest to the focal individual based on Standard Euclidean
800 genetic distance) (Beretta & Santaniello, 2016) (Figure 6). The focal individual will be
801 drawn toward its nearest neighbour, typically an individual within the same population.
802 This method has the advantage of filling missing values even where all individuals
803 collected from a single location are missing for a given locus.

804 If imputation is not desirable or heavy filtering on call rate considered too wasteful of data,
805 an alternative approach is to apply pairwise deletion of loci rather than the global deletion
806 dictated by classical PCA. This can be done by calculating a matrix of Standard Euclidean
807 distances for individuals taken pairwise, after removing loci with a missing value for one or
808 both individuals. Principal Coordinates Analysis (PCoA) can then be applied to the distance
809 matrix to deliver the ordination. This approach capitalizes on the observation that PCA and
810 PCoA, using Standard Euclidean distance, yield the same visualizations (Cox & Cox,
811 2001:43-44). There are cryptic implications of this approach, not least of which is the
812 disruption of the metric and/or Euclidean properties of the distance matrix, so the resultant
813 eigenvalues should be examined for negative values. Negative eigenvalues are unlikely
814 unless the frequency of missing values is extreme.

815 **Linkage**

816 Distance analyses usually assume that each locus contributes independent information to the
817 overall distance value (i.e. they segregate independently). With the large genotype arrays
818 typical of SNP datasets, some SNP loci are likely to be linked (Waples et al., 2022). In

extreme cases, linkage disequilibrium can seriously distort the genetic structure, confounding interpretation. Large blocks of sequence with limited haplotype variation (presumably a result of limited recombination) have been observed in humans (Daly et al., 2001; Patil et al., 2001) and plants (Battlay et al., 2022). If many markers have been sequenced in such a haploblock, they will be tightly linked, and support for any population structure that they represent will be proportionately inflated. This will be evident in a PCA or PCoA as artifactual structure that will potentially defy explanation when working with organisms with little genomic information. An example of such artifactual structure in a PCA plot generated from linked SNP markers is provided by polymorphism in a large inversion in human chromosome 8. SNP markers associated with this inversion generate a coordinated signal which manifests as a three-group pattern, one corresponding to inverted homozygotes, one corresponding to heterozygotes and one corresponding to non-inverted homozygotes (Amos & Ma, 2012; Battlay et al., 2022). Such an explanation was invoked to explain the disaggregation of individuals of the Australian dragon *Pogona vitticeps* into two mirrored clusters that could not be explained by location of capture, sex effects or batch effects (Wild et al., 2022). The signature three-group pattern characteristic of a large polymorphic inversion was evident also in a SNP study of genetic variation across swamps occupied by the Australian Blue Mountains Water Skink *Eulamprus leuraensis* (Figure 8).

It is wise as a data quality step to prune groups of SNPs with high linkage disequilibrium (LD). Unfortunately, the effectiveness of identifying LD is compromised by small numbers of individuals sampled per locality typical of population genetics studies; geographic structure can contribute artificially to estimates of LD. In extreme cases of linkage arising from SNPs residing on a shared, non-recombining block in the genome (Figure 8) can be resolved by identifying loci that are strongly correlated with the Principal Component that separates out the artifactual groupings and removing those loci from the analysis (Wild et al., 2022).

Relatedness among individuals

Representing distance between populations on the basis of a sample typically assumes that is the individuals are sampled at random and their genotypes are independent. However, systematic errors can arise if some sampled individuals are more closely related than are individuals selected at random from their population; these individuals can be expected to separate out from the main body of individuals in a PCA or PCoA (Figure 9a). This can occur

if, for example, individuals are selected from a single school of fish that may be more closely related than individuals chosen at random. The effect can be pronounced if parents and their offspring (siblings) are among the sampled individuals (Figure 9a). These closely related individuals should be identified and all but one removed from the analysis.

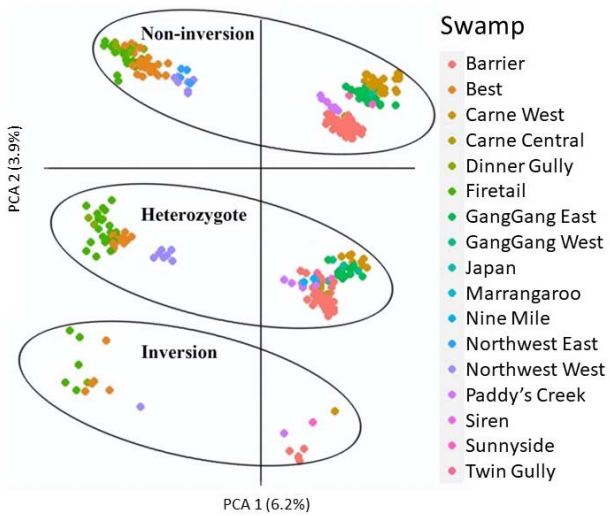

Figure 8. An ordination of SNP genotypes for the Australian Blue Mountains Water Skink *Eulamprus leuraensis* collected from 17 locations with varying levels of isolation. The ordination shows the three-group structure characteristic of a large autosomal inversion (Amos & Ma, 2012). For sake of illustration, we have assumed that the inversion is the least frequent of the two polymorphic states. This interpretation remains speculative until sufficient genomic information is available for this species to demonstrate the existence of the inversion and to associate the SNP loci strongly correlated with PCA 2 to that inversion.

When comparing two or more populations, relatedness among sampled individuals to a greater degree than among individuals in their population as a whole will potentially affect the estimate of genetic distance between that population and others. This can lead to misinterpretation by artificially displacing the affected population from what otherwise would be a spatio-temporal trend. An important assumption of these analyses is that the sampled individuals are drawn at random from their populations and that their genotypes are independent. Datasets should be screened and filtered for close familial relationships in studies of spatio-temporal genetic variation at the level of populations. Care should also be

exercised when interpreting clinal structure if the unit of dispersal comprises groups of related individuals (Fix, 1997).

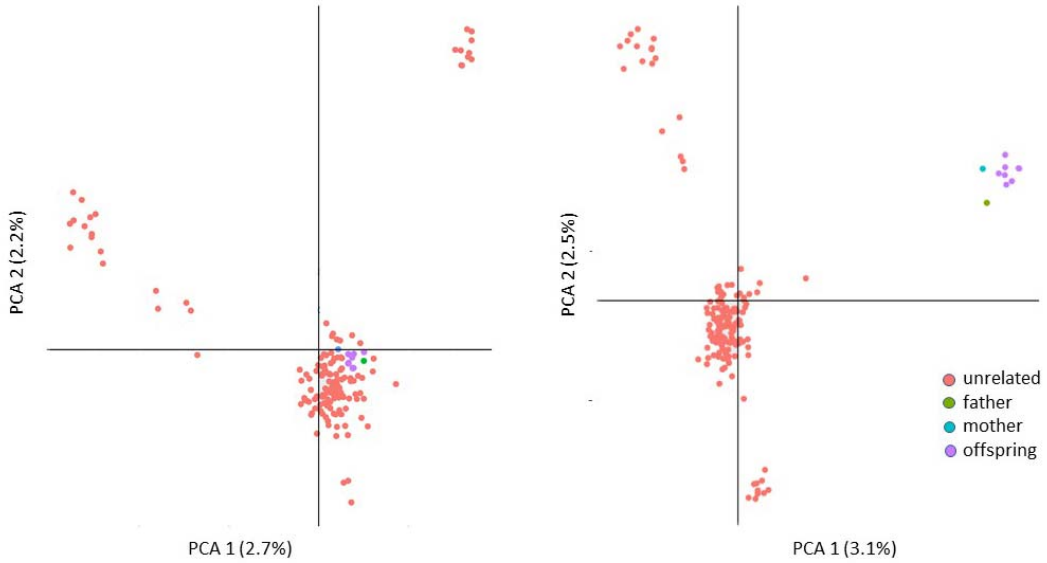

Figure 9. A series of sub-populations of *Emydura macquarii* from the northern basin of the Murray-Darling drainage (a) in which one subpopulation (central) has two individuals identified to be manipulated artificially into a parent-offspring relationship and eight individuals into a parent-offspring relationship; (b) The ordination after the addition of the modified individuals. If such closely related individuals go undetected, and are retained, the spatio-temporal distance relationships between populations can be subject to misinterpretation.

Discussion

Species and populations usually do not constitute a single panmictic unit where individuals breed at random over their entire range. Population subdivisions typically exist, which may be hierarchically arranged if they reflect patterns of ancestry and descent, or not if they are each on independent random walks, perhaps periodically reset by episodic gene flow (Georges et al., 2018). It follows that distances between entities, be they individuals, aggregations of individuals, populations or taxa, will often be highly structured. The challenge for SNP analyses arises from dealing with an incredible volume of data, certainly in the thousands of markers but often in the hundreds of thousands or even millions of markers each with its own contribution to overall variation. Summarising the data as a distance matrix is a first step in examining structure.

894 The applications of genetic distances calculated from SNP data are diverse, because
895 substructuring of populations arises from interesting demographic and biogeographic
896 processes or conversely because such substructuring, if undetected, can confound analyses
897 that assume no such substructuring. On the latter point, human genomic studies, where
898 contributions of alleles to a phenotype are statistically measured using genome-wide
899 association studies, require samples to be from one population without any substructure
900 (Uffelmann et al., 2021). However, this is rarely the case given that self-reported ancestries
901 and definition of social groups do not always capture the underlying genetics owing to
902 admixture resulting in continuous population structure. Genetic distance measures and their
903 ordination using the continuous variation from PCA axes can be a powerful way to adjust
904 genotypes and phenotypes based on ancestry to compute association statistics (Tian et al.,
905 2008). With regard to contributions to our understanding of demographic and biogeographic
906 processes, one area that has received substantial attention, is isolation by distance. Isolation
907 by geographic distance (Wright, 1943) in a homogenous and continuous landscape is perhaps
908 the simplest instance of departure from panmixia in a widespread population, and is typically
909 examined by comparing a genetic distance matrix with geographic distance between
910 individuals, sub-populations or sampling localities. Fst and Chord Distance are often chosen
911 as the measures of genetic distance to examine any relationship with geographic distance
912 (Séré et al., 2017). Such a relationship is often used as the null hypothesis against which to
913 examine more complicated hypotheses to explain patterns of variation across the landscape
914 (Manel et al., 2003; Spear et al., 2015). In particular, isolation by distance can be complicated
915 by variation in resistance to gene flow in complex landscapes (McRae, 2006). Introduction of
916 the concept of landscape resistance delivers a continuous set of circumstances between
917 genetic structure arising from isolation by distance in otherwise freely breeding populations,
918 and subdivision between populations of a species where gene flow is absent, rare or an
919 episodic event. More novel applications of genetic distances include stratified sampling of a
920 core collection of specimens from a larger pool of accessions to maximally capture genetic
921 diversity (Rogers D: Jansen & van Hintum, 2007) and tracking the relationships of SARS-
922 CoV-2 variants (Jaccard D: Yin, 2020).

923 A second area of endeavour that has benefitted from the advances afforded by SNP
924 markers and their associated distance measures is phylogeography, both in the context of
925 human populations (Pugach & Stoneking, 2015; Tassi et al., 2015) and animal and plant
926 populations (S. V. Edwards et al., 2015). Here, genetic substructuring is mapped onto the

927 landscape and interpreted in the context of historical (Georges et al., 2018) and contemporary
928 (J. L. Mijangos et al., 2022) barriers to geneflow. SNPs and distances derived from them are
929 also useful in population assignment (Negrini et al., 2009) and for putatively identifying
930 migrants within source populations (Mamoozadeh et al., 2020). These analyses rely on
931 underpinning by genetic distances and their visualization.

932 Many distance measures are available for different types of genetic data (Kosman &
933 Leonard, 2005; Libiger et al., 2009). Fortunately, characteristics of SNP data and considering
934 contemporary rather than historical processes governing variation among populations,
935 dramatically reduce the options from which to select an appropriate distance. SNP markers
936 are biallelic in practice, so distinct distance measures in a multiallelic context can become the
937 same in a biallelic context. Additionally, symmetry considerations in the arbitrary choice of
938 reference and alternate allele eliminate from use distance measures that treat scores of
939 homozygous reference (0) as fundamentally different from scores of homozygous alternate
940 (2). Because genetic distances are likely to be interpreted in the context of everyday notions
941 of a distance measure, even if subconsciously, distances that satisfy the basic metric
942 properties are preferable over those that do not. The decision then comes down to one of
943 context.

944 Genetic structure within and among populations is commonly explored using
945 ordination which is inherently reliant on the concept of genetic distance. Strictly, distances
946 with Euclidean properties are required to faithfully represent those distances in a space
947 defined by Cartesian coordinates using PCA and PCoA. Distances with metric properties
948 yield adequate representations in most cases (e.g. Jaccard D, Manhattan D, Reynolds D,
949 Chord D). Non-metric distances should be avoided. However, methods for detecting
950 distortion of a visual representation arising from gross departures in Euclidean and non-
951 metric properties are available (negative Eigenvalues) as are means for redressing the
952 deficiency. In practice, the distortion arising from selecting only the top two or three axes for
953 the visualization generates more distortion than does a relatively few negative eigenvalues.
954 One can thus be somewhat relaxed about departures from Euclidean or metric properties of a
955 distance if there are compelling reasons for choosing a non-metric distance measure in the
956 context of the study and questions to be explored.

957 PCA has recently come under criticism (Elhaik, 2022). The deficiencies of PCA
958 identified by Elhaik are real, but we argue that they derive from over-interpretation of PCA to

959 address questions that are beyond its remit, and because of misunderstanding of what
960 ordination delivers as its ultimate solution. PCA is essentially an exploratory tool that draws
961 out patterns from multivariable data by aggregating correlated influences into a relatively few
962 dimensions. PCA is about extracting patterns. It is little to contribute to understanding of the
963 processes that delivered those patterns. The patterns thus are suggestive but not
964 demonstrative of underlying causes. Interpretations of the causes of the patterns need to be
965 pursued by more definitive subsequent analysis. For example, individuals that fall in an
966 intermediate position between two well defined groupings in a bivariate plot of PCA1 and 2
967 may be an indication of admixture. Certainly, that is the position in the ordination plot that
968 admixed individuals will fall, but the reverse inference is not true. The intermediary position
969 may not be sustained on examination of deeper dimensions or may have arisen from other
970 causes. Definitive demonstration of admixture requires additional analyses (refer Figure 4 of
971 Georges et al., 2018), such as the frequency of heterozygotes at loci fixed and different for
972 the two major groupings, or use of statistical packages such as NewHybrids (Anderson &
973 Thompson, 2002). Similarly, PCA may be undertaken as a prelude to genome-wide
974 association studies (GWAS) to identify genes under selection (Xu et al., 2022). Serious
975 difficulties arise in terms of defensible and reproducible conclusions when interpretation is
976 restricted to PCA without targeted and appropriate analyses to follow the indications from the
977 PCA. One cannot reliably use two or three-dimensional representations in PCA alone to
978 decide ancestry or demographic history, to reliably identify and exclude outliers, to infer
979 kinship, to identify ancestral clines in the data, to detect genomic signatures of natural
980 selection, or to identify cases of convergent evolution (Elhaik, 2022 and references cited
981 therein). PCA can provide only suggestive indications useful for formulating hypotheses for
982 further rigorous examination. Therein lies its value.

983 A second criticism of PCA is that the visual representation of distances between
984 individuals is sensitive to sample size (Elhaik, 2022). This sample size dependency arises
985 because increasing the sample size of one structural grouping (population, aggregation of
986 populations, taxon) will reorient the individuals in the space defined by the informative
987 dimensions of the ordination. This leads to potentially dramatic changes in the plot of the top
988 two dimensions. Obviously, this sample size effect will have major consequences for over-
989 interpretation of two or three-dimensional PCA plots. So the apparent lack of reproducibility
990 identified by Elhaik (Elhaik, 2022) arises from restricting attention to only the top two (or

991 three) of more informative dimensions. Sample size does not impact on the reproducibility of
992 the ordinated solution provided all substantial informative dimensions are considered.

993 Arguably, missing values, and how they are managed, is the issue with the most
994 significant consequences for genetic distances and their visualisation. Classical PCA requires
995 a complete input dataset, and adequate filtering missing values requires removal of loci or
996 individuals from the analysis to create the input matrix free of missing values. For SNP data,
997 which typically have high frequencies of missing scores, the consequent data loss is
998 unacceptable. To overcome unacceptable loss of data, the missing values are typically infilled
999 using the global average for the locus concerned. We have shown that this can result in
1000 misleading displacement of individuals with high rates of missing data away from their
1001 natural groupings and toward the global centroid, with the risk of misinterpretation. It can
1002 also inflate confidence envelopes for aggregations of entities in a PCA, with serious
1003 consequences for analyses such as population assignment. We have reviewed the approaches
1004 to deal with missing values that avoid these distortions, the most effective of which appears
1005 to be replacement of a missing value with that of the nearest neighbour (Beretta &
1006 Santaniello, 2016). Even replacement with a random value is preferable to replacement with
1007 the global average allele frequency (the most common default approach) because random
1008 values simply add noise to the data which is driven to lower dimensions in any ordination.

1009 Missing values can be managed on a pairwise basis, noting that PCA and PCoA with
1010 Euclidean Distance yield the same outcome. PCoA is less affected by missing values because
1011 they are eliminated from the computations taking the entities a pair at a time. However,
1012 PCoA is not immune to distortion from missing values, as their presence destroys the metric
1013 and Euclidean properties of the distance measure, and therefore the ability of PCoA to
1014 faithfully represent the distance matrix in a space defined by Cartesian coordinates. There are
1015 also issues with the variance of distances calculated locus by locus when the number of
1016 values differs among loci because of missing values. Other concerns are linkage, which is
1017 difficult to detect and filter when sample sizes from collection localities are small, because
1018 genetic structure among collection localities contributes artificially to estimates of linkage
1019 disequilibrium. In gross cases of linkage, such as those arising from a polymorphic
1020 chromosomal inversion (Figure 8), SNPs associated with the inversion can be identified and
1021 removed (Wild et al., 2022).

In summary, we recommend selection of a distance measure for SNP genotype data that does not give differing outcomes depending on the arbitrary choice of reference and alternate alleles, and careful consideration of which state should be considered as zero when applying binary distance measures to SNP PA data. As a broad recommendation on choice of a distance measure, Standard Euclidean Distance is the default choice of a genetic distance for SNP data. It is free of assumptions about the processes that generate the variation and, in the biallelic case, does not suffer the unacceptable distortions that arise in multiallelic data when two populations are polymorphic at a site, but share no alleles (Nei & Kumar, 2000:246). Standard Euclidean Distance can be faithfully represented in ordinated Cartesian space. Other metric distances may be chosen in order to down weight joint absences in the case of SNP PA data (e.g. Jaccard Distance) or because of the importance of a particular model of progressive divergence with time (e.g. Nei's D, Reynold's D, Chord D). For example, Chord Distance is better correlated with time since divergence and is a metric distance, so may be a better choice than Fst /(1- Fst) (a non-metric distance) when analysing isolation by distance (Séré et al., 2017). Diagnostic examination of eigenvalues should be undertaken when a non-metric distance has been selected, or if the analysis is to include substantial missing values. Action should be taken if the sum of negative eigenvalues is substantial, to avoid distortion in the final visual representation. We strongly recommend filtering heavily on missing values, then imputing those that remain to create a full matrix prior to undertaking a distance analysis. Failure to do so can substantially and artificially inflate confidence envelopes for populations or aggregations of populations and lead to other distortions that can lead to misinterpretation. Screening for closely related individuals (parent-offspring or sibling relationships) is also important, and the impact of polymorphic haploblocks in the genomes of target species or populations occasionally emerges as a challenge for studies of species with limited genomic information.

Data Availability

Real datasets and the scripts used to generate simulated datasets used in this paper are available on Dryad (URL to be provided on acceptance).

Author Contributions

All authors contributed to the development of ideas presented in this manuscript. AG led the writing, LM and BG undertook the simulations, HP provided input on human studies, MA provided access to the skink data and provided associated context.

Acknowledgements

We would like to thank Bill Sherwin, Fred Allendorf, Evsey Kosman and an anonymous reviewer for comments on an earlier draft of this manuscript, and for assisting with setting the scope and direction of the manuscript. We would like also to thank Springvale Coal Pty Ltd for allowing the publication of genetic data obtained for the Blue Mountains Water Skink Research and Management Program coordinated by RPS Australia East Pty Ltd for the Springvale Extension Project.

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