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Analysis of haplotype networks: the randomized minimum spanning tree method

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Summary

1. Haplotype network construction is a widely used approach for analysing and
3 visualising the relationships among DNA sequences within a population or
species. This approach has some problems such as how to quantify alternative
links among sequences, or how to plot efficiently networks to compare them easily.
- 6 2. In this paper, a new method is presented: the randomized minimum spanning tree
method, based on randomizing the input order of the data in order to produce
alternative branchings in the haplotype network. It is shown that this new method
9 can produce, at least in some situations, networks with less alternative links than
the minimum spanning network method.
- 12 3. A new graphical display of haplotype networks is introduced here. This is based
on calculating the coordinates of the haplotypes from a multidimensional scaling
of the haplotype distance matrix. The display can be done in two or three
dimensions. The eigenvalues extracted from the multidimensional scaling analysis
15 give an indication of the relevant number of dimensions.
- 18 4. These tools are illustrated with the analyses of published data on the leopard and
on the jaguar. These analyses show interesting and contrasting patterns between
these two species of big cats.
5. All tools are implemented in R and available in the package pegas.

Keywords: Hamming distance, haplotype network, microevolution, minimum spanning
21 tree, *Panthera*

Introduction

The analysis of DNA sequences from individuals sampled in one or several populations
24 makes possible to address different questions on microevolutionary processes such as
population structure, gene flow, past demographic bottlenecks and expansions, or
geographical events of colonizations and extinctions (Bossart & Prowell, 1998; Emerson
27 *et al.*, 2001; Buerkle & Lexer, 2008). An important aspect of these analyses is the
inference of ancestry–descendance relationships among haplotypes. Typically, if the
sequences are assumed to be contemporaneous, two alternative approaches can be
30 adopted: inferring either a phylogenetic tree, or a network. The phylogenetic approach
assumes that the ancestral sequences are unobserved and associated with the internal
nodes of the tree while the observed sequences are associated with its terminal nodes.
33 On the other hand, if some observed sequences may be ancestral to others, a network
approach is more appropriate, in which case these sequences will be associated with the
internal nodes of the network. It is clear that choosing an approach or the other will
36 depend on the time frame and the mutation rate of the sequences. If the sequences are
sampled sequentially through time (e.g., pathogens sampled through an epidemic, or
ancient DNA), several approaches have been developed to take this temporal dimension
39 into account (e.g., Jombart *et al.*, 2011).

Network methods can be classified according to different criteria (Table 1). For
instance, it is possible to define two categories depending on their main objective: the
42 ‘explicit’ networks depict reticulated processes of evolution such as hybridization,
horizontal gene transfer, or admixture, whereas the ‘implicit’ networks represent some
form of uncertainty over a tree (i.e., without reticulation) representation (Huson &
45 Bryant, 2006; Kloepper & Huson, 2008). Another way to classify network methods
considers the reconstruction method: these include parsimony, distances, maximum
likelihood, Bayesian inference, split decomposition, or consensus methods (Holland

48 *et al.*, 2004). Another important criterion is whether unobserved haplotypes can be
included in the network: distance-based methods cannot generally do this because they
do not consider explicitly the evolving characters (Table 1). It is crucial to assess the
51 reliability of a phylogeny or network of haplotypes since its construction may be affected
by sampling biases (i.e., missing haplotypes). A badly estimated network is likely to lead
to wrong inference of population or history.

54 The present paper focuses on the distance-based, implicit network approach. Two
methods are commonly used to build a haplotype network: the minimum spanning tree
(MST) method (Kruskal, 1956) and the method from Templeton *et al.* (1992, TCS). The
57 MST method has been applied in many fields (Nešetřil *et al.*, 2001). Its principle is to
first build a matrix of pairwise distances among sequences (or haplotypes), and then find
the shortest set of paths that link all observations where the length of each link is taken
60 from the pairwise distance. The TCS method, often referred to as statistical parsimony,
is based on a model of evolution of the genetic characters measured on each
individual—originally restriction fragment lengths but the method can be applied to
63 DNA sequences. The main difference between both methods is that an MST is a network
with no reticulation, thus for n sequences, the resulting MST will have $n - 1$ links. On
the other hand, a TCS network may have reticulations defining alternative branchings,
66 and include unobserved haplotypes in the network. Thus, this method may be used to
infer micro-evolutionary events such as putative recombinations (Posada & Crandall,
2001). Bandelt *et al.* (1999) developed another method called the median-joining
69 network (MJN) where alternative branchings are found by examining potential ancestral
sequences for each triplet of sequences. MJN belongs to a class of methods which
includes several variants such as reduced median network or quasi-median network
72 (Bandelt *et al.*, 1995, 1999). Thus, the MST usually cannot represent possible
ambiguous or alternative branchings. Another limitation of the MST construction is that,

for a given data set, the MST may not be unique. Figure 1 shows a simple illustration of
75 this problem: the three represented data sets are identical but the inferred MSTs are
different because the observations are ordered differently. This is a consequence of the
presence of ties in the distance matrix (see the algorithm descriptions below). With real
78 data, this problem and its consequences may be very hard to detect with a large number
of observations. Bandelt *et al.* (1999) pointed out that instead of constructing a single
tree, it is possible to construct a minimum spanning network (MSN) by modifying
81 slightly Kruskal's (1956) algorithm as explained below.

In this paper, I propose a new algorithm to construct a network which can be seen as
intermediate between the MST and MSN methods. I also present tools to compare
84 networks programmed in R (R Core Team, 2017).

Methods

MINIMUM SPANNING TREE AND NETWORK

The MST algorithm can be sketched as follows (Kruskal, 1956):

- 87 1. Compute the matrix of pairwise distances among the n observations and sort them
in increasing order.
2. Assign each observation to its own group; there are thus n initial groups.
- 90 3. Set $i \leftarrow 1$.
4. Take the i th distance from step 1: if the two corresponding observations are not in
the same group, then create a link between them and pool the two groups.
- 93 5. Set $i \leftarrow i + 1$.
6. Repeat steps 4 and 5 until there is a one group.

The number of groups is decreased by one at each iteration of step 4. The issue with this
96 algorithm is how ties in the distance matrix are treated in step 1, and this is, obviously,
dependent on the implementation. The MSN tries to solve this problem (Bandelt *et al.*,
1999); its algorithm is:

- 99 1. Compute the matrix of pairwise distances among the n observations, extract the
unique values, and sort them in increasing order (denoted as $\delta_1, \delta_2, \dots$).
2. Set $i \leftarrow 1$.
- 102 3. Create the links for all pairs of observations with distance equal to δ_i .
4. If all observations are linked in a single group, then stop.
5. Set $i \leftarrow i + 1$, and go to step 3.

THE RANDOMIZED MINIMUM SPANNING TREE

105 The input data are a set of aligned sequences from which a distance matrix is computed.
The sequences could be DNA or other kinds as long as there is a method to compute
pairwise distances. In most applications, a simple Hamming distance (or Manhattan in
108 the case of binary characters) will be relevant. In order to remove the influence of the
order of the input data, the procedure is based on a randomization of this order. This is
repeated many times and for each replication an MST is constructed. The MSTs are then
111 post-processed in order to return a single network including all the links observed among
the replications. Because of the nature of the proposed algorithm, it is called here the
randomized minimum spanning tree (RMST) method.

114 The RMST usually has less links than the MSN. Figure 2 shows a simple example
with four binary sequences. The first step of the network construction is to consider links
of length one A–C and A–B; the second step considers the links of length two A–D and

117 B–C. During the MST construction, the link B–C is never included because B and C
were already grouped together during the first step, so the RMST does not include this
link. On the other hand, the MSN includes this link thus resulting in two alternative
120 paths, B–A–C and B–C, both of the same length. The RMST avoids this ambiguity. Note
the difference between the present example and the one in Figure 1: in the latter, the
additional link output by the MSN creates a path shorter than the one created by the
123 MST.

GRAPHICAL TOOLS

Plotting networks is a notoriously difficult problem (e.g., Klopper & Huson, 2008).
Several computer programs perform graphical display of various types of evolutionary
126 networks, such as igraph (Csardi & Nepusz, 2006), network (Butts, 2008), phangorn
(Schliep, 2011), pegas (Paradis, 2010), or SplitsTree (Huson, 1998), among many others.
In practice, it would be very useful to graphically compare networks constructed under
129 different methods or assumptions. However, this is usually not possible (or very difficult)
because there is no standard procedure for plotting haplotype networks. To propose a
solution to this problem, an implementation based on multidimensional scaling (MDS;
132 Torgerson, 1952) is developed here. The procedure is to first perform an MDS on the
distance matrix in order to extract two or three sets of coordinates. These coordinates are
then used to plot the observed sequences or haplotypes in 2-D or in 3-D, and the links
135 inferred from the network are then drawn. Thus, this procedure contrasts with most
existing ones which compute the layout of haplotypes trying to minimize line crossings
(e.g., Klopper & Huson, 2008). The proposed procedure has several advantages. First,
138 an MDS on the original distance matrix will arrange the sequences depending on their
similarity. Second, the eigenvalues extracted from the MDS make possible to assess
whether it is relevant to use two or three dimensions in this projection. Third, the

141 coordinates of the observations will be the same for all networks since they depend only
on the distance matrix, making graphical comparisons easier. Fourth, the procedure is
computationally straightforward since an MDS is usually fast to perform even with
144 several hundreds observations.

The tools presented in this article have been implemented in the R package *pegas*
(Paradis, 2010). This package has already implemented a standard 2-D plot using an
147 “energy-minimisation” algorithm to optimise the layout. Plots in three dimensions have
been implemented using the *rgl* package (Adler *et al.*, 2016).

SIMULATION STUDY

To assess how the RMST is able to find alternative links in a network of haplotypes,
150 some simulations were run under different situations of mutation rate (μ), sequence
length (l), and number of sequences (n). A set of n sequences was simulated under the
JC69 model of sequence evolution (Jukes & Cantor, 1969) along a random network with
153 no reticulation and link lengths taken from a standard uniform distribution. The network
was simulated by generating a random binary tree where the internal nodes were
considered contemporaneous to the leaves. The sequences were then analysed with the
156 RMST using different numbers of randomizations (5, 10, 20, 50, 100): the number of
additional links found by the RMST as well as the number of unique distances were
recorded. The other parameters were: $n = 50, 100, 500, \text{ or } 1000$, $l = 500 \text{ or } 1000$, and
159 $\mu = 0.01 \text{ or } 0.1$. These parameter values were chosen to result in substantial numbers of
ties in the distance matrices. The simulations were replicated 100 times for each
combination of n , l , and μ . The code used is provided in the Supplementary Information.

DATA

162 Two data sets were considered to apply the methods introduced in this paper: a set of
mtDNA sequences from 33 leopards (*Panthera pardus*) published by Uphyrkina *et al.*
(2001), and a set of mtDNA sequences from 37 jaguars (*P. onca*) published by Eizirik
165 *et al.* (2001). Both data sets were downloaded from GenBank (accession numbers:
AY035227–AY035292 and AF244814–AF244887, for each species, respectively). The
sequences were aligned separately for the different genes (using information from
168 GenBank and from the original publications) with MUSCLE (Edgar, 2004), and then
combined into two global alignments with 726 and 707 sites, respectively. All sequences
were unique for the leopard data, but 22 unique sequences were identified for the jaguar
171 data. For each alignment, a matrix of Hamming distances was calculated with *ape*
(Paradis *et al.*, 2004). These matrices were used as input for the construction of the
networks. The individual labels from the original studies were kept for the present
174 analyses. The R scripts used for these analyses is provided in the Supplementary
Information.

Results

SIMULATION STUDY

177 To simplify the presentation of the results, the number of additional links was calculated
as successive differences with increasing number of randomizations (i.e., the numbers of
links found with five randomizations compared to zero, with ten randomizations
180 compared to five, and so on). The results were clearly related to the number of unique
distances among the simulated distances. Considering that the total number of distances
is given by $n(n-1)/2$, the percentage of unique distances was always less than 1%
183 (Table 2). Increasing l and/or μ resulted in more variation among the sequences and,

consequently, less additional links for the same n . On the other hand, increasing n for the same values of l and μ resulted in more closely related sequences, and thus more
186 additional links found by the RMST. In three cases, some additional links were still found with 100 randomizations. In the scenario simulating the most diversity ($l = 1000$, $\mu = 0.1$), five randomizations were enough to find the additional links of the RMST for
189 all values of n .

APPLICATION

The MDS analysis of the distance matrices resulted in slightly different patterns of eigenvalues. For the leopard data, the first eigenvalue was much larger than the others
192 though the second and third ones were substantially larger than the remaining ones (Fig. 3a). For the jaguar data, the first and the second eigenvalues were much larger than all the other ones (Fig. 3b). Thus, we may anticipate that three dimensions may represent
195 the distribution of sequences for the leopard data whereas two dimensions may be enough for the jaguar data.

The MSN and RMST analyses revealed large numbers of additional links compared
198 to the MST ones (Table 3). In the case of the leopards, the number of links was multiplied by 6.7 from the MST to the MSN, and by 1.9 to the RMST. This increase in number of links was slightly smaller in the case of the jaguar: 5 to the MSN and 1.3 to
201 the RMST. The RMST analyses were repeated with different numbers of randomizations. For the leopard data, 59 links were found with 10 randomizations while 60 links were found with 50 randomizations or more. For the jaguar data, 28 links were
204 found with 10 randomizations or more.

The MDS-based plots of the leopard sequences showed a clear continental separation with the African individuals (SHO*) on the right-hand side of the plot, and the Asian
207 ones on the left-hand side (Fig. 4). Interestingly, two individuals laid outside of these

two groups: the one from the Arabian Peninsula (NIM1) and the one from Java, Indonesia (MEL1). Remarkably, the RMST did not add any further link from NIM1 to
210 the others, whereas two additional links were observed between MEL1 and the Asian ones. The MSN kept NIM1 with a single link to SAX2, but added one further link between MEL1 and the others. The 3-D displays revealed that the African individuals,
213 which are apparently aligned in the 2-D plots, are actually arranged along an arc in the third dimension of the MDS (videos provided in the Supplementary Information).

For the jaguar data, the arrangement of the individuals on the first axis followed a
216 North–South axis with individuals from the South on the right-hand side of the plot (Fig. 5). Two individuals remained single-linked with the MSN and RMST analyses: Pon23 from Nicaragua which was linked with two individuals from Nicaragua and from
219 Costa Rica, both with the same haplotype, and Pon63 from Venezuela which was linked with Pon73, an individual of unknown origin but presumably from Brazil (Eizirik *et al.*, 2001). Except for Pon63, very little dispersion was observed in the third dimension as
222 expected from the eigenvalues of the MDS (videos provided in the Supplementary Information).

Discussion

225 The analysis of the relationships among DNA sequences and haplotypes within and among populations is crucial for testing hypotheses on microevolutionary processes. However, such analyses often suffer from shortcomings. Typically, two issues are often
228 observed. First, practitioners usually construct a single haplotype network which is then interpreted depending on the context of the study. This can be a problem when the assumptions of the method are not met, which can usually be assessed by comparing
231 different constructions, for instance, by using different distances. The second problem is that there seems to be a confusion in the literature between MST and MSN. It is common

to read “minimum spanning network” when an MST is obviously shown since no loop is
234 present. This is problematic since the MST may not be unique as already mentioned by
Bandelt *et al.* (1999).

The RMST method is an alternative to the MSN with the advantage of creating less
237 links among haplotypes while fully taking into account the ambiguities induced by data
ordering. In the applications with real data presented in this paper, 100 randomizations
were done and the results were identical than with smaller numbers. The computing
240 times of the method is thus proportional to the product of n (since $n - 1$ links are built at
each replication of the MST algorithm) with the number of randomizations. The
implementation in `pegas` resamples the distance matrix by reordering its rows and
243 columns simultaneously, instead of reordering the original data matrix, and thus avoids
to recalculate the distance matrix at each iteration of the MST (which requires a
computing time proportional to n^2).

246 The simulation study showed that the number of randomizations required to reach
convergence of the RMST procedure is affected by the number of sequences, the
sequence length, and the mutation rate. Because it is not easy to define a priori how
249 many randomizations are required for a given data set, it is recommended to repeat the
analyses with increasing numbers of randomizations and check that the constructed
networks are identical (see code in Supplementary Information).

252 The respective merits of the methods used to construct haplotype networks are still
debated (e.g., Mardulyn, 2012). However, comparing different methods is not without
difficulties because some of them construct networks that have inherently different
255 structures. Parsimony-based methods seek to combine all most parsimonious
phylogenetic trees into a network which, as a consequence of this estimation procedure,
have the observed sequences only at its terminal nodes (Branders & Mardulyn, 2016).
258 This contrasts with, on one hand, the MST-based methods where the observed sequences

are at both the internal and the terminal nodes of the network, and, on the other hand, the TCS or MJN method which includes unobserved sequences in the network. For instance, 261 the MJN constructed with the data in Figure 1 would have four nodes and three links all of length one, but this network is not strictly different from the MSN and RMST ones (Fig. 1c) because they all define paths of length two between each pair of observed 264 sequences. Clearly, the presence of loops in RMST or MSN networks must be interpreted cautiously with respect to potentially unsampled haplotypes (e.g., Joly *et al.*, 2007). On the other hand, the MJN inferred from the data in Figure 2 would be identical 267 to the RMST (Fig. 2d) but different from the MSN (Fig. 2c).

Plotting networks (i.e., graphs with reticulations) is notoriously difficult for graphical software developers. This is another difficulty in the analysis of haplotype relationships. 270 The graphical approach proposed here is a solution to this problem. By using the coordinates inferred from the MDS applied on the distance matrix, the haplotypes are always positioned in the same way, for a given distance matrix, whatever the links 273 among them. Furthermore, the analysis of the eigenvalues extracted from the MDS gives information on the general structure of the data as illustrated by the examples above.

The analyses of the leopard and the jaguar data were mainly illustrative, although 276 they show some interesting results. For the leopards, the contrast between African and Asian individuals was very clear. Two individuals were outside the bulk of the other individuals: one from the Arabian Peninsula, and the other from Java. Both represent 279 two subspecies (*P. pardus nimr* and *P. pardus melas*) that are morphologically markedly different from the others (Stein & Hayssen, 2013). Variation within the African group was also substantial and appeared in the third dimension of the plot. For the jaguars, 282 variation was much less than for the leopards, and the MSN and the RMST showed much more additional links than the MST. Interestingly, Eizirik *et al.* (2001) reported an MSN with only one additional link. However, the present analysis showed that the

285 RMST had seven additional links and the MSN even more.

Another interesting result is the presence of a “horseshoe effect” with the leopard data but not with the jaguar data. This effect, which is sometimes observed in
288 multivariate analyses like MDS or principal component analysis (PCA), is a consequence of the dominance of local structures in the data. Such dominance can be the result of the inherent structure of the original data matrix (Ahmed *et al.*, 1974), a
291 transformation of the distances that gives more emphasis on the most similar observations (e.g., an exponential decay function; Diaconis *et al.*, 2008), or, typically for population genetic data, local processes such as isolation by distance (Novembre &
294 Stephens, 2008). Whatever the origin of such structures, the decomposition of the data matrix (in the case of PCA) or of the distance matrix (in the case of MDS) results in the second axis to be related to the first one in a polynomial-like manner (actually a
297 sinusoidal function; see Ahmed *et al.*, 1974; Diaconis *et al.*, 2008), and the subsequent axes with increasing degrees of the polynomials. In practice, the proximities of the sequences on the second and third axes must therefore be interpreted with caution.
300 However, this does not affect the interpretation of the network layouts which are the same for all networks as long as the distance matrix is the same.

As rightly pointed out by Leigh & Bryant (2015), the haplotype network
303 methodology does not generally rely on an evolutionary model. However, a distance-based approach is very valuable because distances can be computed for different kinds of data, and they are straightforward to interpret in terms of number of changes.
306 An interesting perspective will be to develop an approach to incorporate models of DNA sequence evolution into haplotype network analyses. A challenge will be to find how to compute a likelihood in the presence of loops in the network (Maynard Smith, 1989).

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Data accessibility

The data are archived in GenBank (accession numbers: AY035227–AY035292 and AF244814–AF244887).

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393 **Supporting Information**

- sim.R.** Code to run the simulations.
- analysis_cats.R.** Code to reproduce the analyses of the leopard and jaguar data.
- 396 **pardus_MST.mp4.** Animation of the MST from the leopard data.
- pardus_MSN.mp4.** Animation of the MSN from the leopard data.
- pardus_RMST.mp4.** Animation of the RMST from the leopard data.
- 399 **onca_MST.mp4.** Animation of the MST from the jaguar data.
- onca_MSN.mp4.** Animation of the MSN from the jaguar data.
- onca_RMST.mp4.** Animation of the RMST from the jaguar data.

Table 1: Comparison of some features of different methods to construct haplotype networks. MST: minimum spanning tree; MSN: minimum spanning network; RMST: randomized minimum spanning tree; TCS: statistical parsimony; MP: maximum parsimony; MJN: median-joining network; n : number of haplotypes; L : number of links in the network.

Method	Input data	L	Unobserved haplotypes	Reference
MST	Distances	$n - 1$	No	Kruskal (1956)
MSN	"	$\geq n - 1$	No	Bandelt <i>et al.</i> (1999)
RMST	"	"	No	This paper
TCS	Sequences	"	Possibly	Templeton <i>et al.</i> (1992)
MP	"	"	Yes, at internal nodes	Branders & Mardulyn (2016)
MJN	"	"	Possibly, as median-vectors	Bandelt <i>et al.</i> (1999)

Table 2: Simulation results: mean number of additional links found by increasing the number of randomizations in the RMST algorithm (l : sequence length; μ : mutation rate; n : number of sequences; NUD: mean number of unique distances).

l	μ	n	Number of randomizations					NUD
			5	10	20	50	100	
500	0.01	50	29.96	7.79	3.24	1.16	0.04	39.11
		100	60.25	15.62	6.78	2.75	0.31	50.40
		500	302.88	74.31	33.65	12.29	1.61	80.24
		1000	613.10	156.06	75.05	28.22	3.44	94.85
	0.1	50	2.48	0.18	0.02	0.00	0.00	216.09
		100	5.71	0.52	0.01	0.00	0.00	270.74
		500	33.26	2.89	0.25	0.04	0.00	340.92
		1000	63.49	4.96	0.38	0.04	0.00	363.05
1000	0.01	50	14.58	1.78	0.68	0.07	0.01	71.25
		100	26.42	3.61	0.79	0.14	0.00	96.87
		500	147.65	23.23	7.15	1.58	0.00	149.60
		1000	292.88	45.67	11.77	2.46	0.16	177.73
	0.1	50	1.33	0.21	0.00	0.00	0.00	390.53
		100	2.74	0.23	0.00	0.00	0.00	520.35
		500	15.37	1.58	0.05	0.00	0.00	671.83
		1000	31.22	2.18	0.11	0.00	0.00	707.06

Table 3: Number of links in the networks constructed with the two data sets analysed. n : number of haplotypes.

Species	n	Number of links		
		MST	MSN	RMST
Leopard	33	32	214	60
Jaguar	22	21	105	28

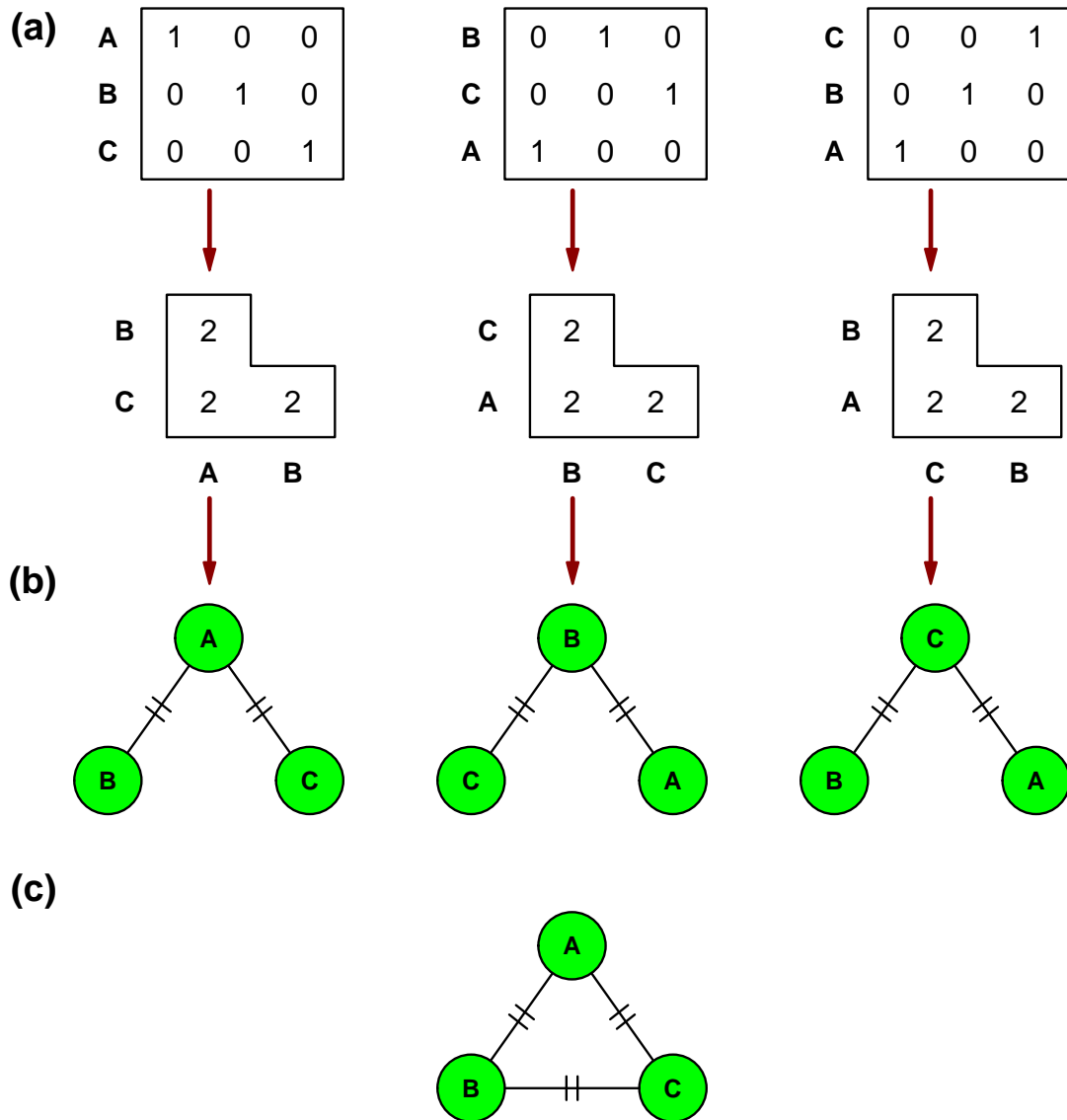


Figure 1: (a) Three identical data sets but with rows in different order and the corresponding distance matrices. (b) The three minimum spanning trees (MST) are different. (c) The minimum spanning network (MSN) and the randomized minimum spanning tree (RMST) are identical for the three data sets.

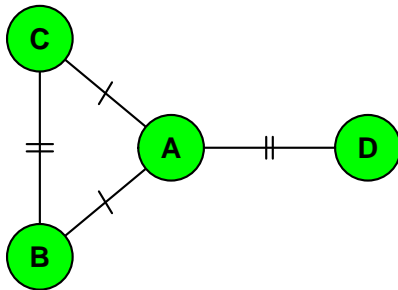
(a)

A	1	1	0	0
B	0	1	0	0
C	1	0	0	0
D	1	1	1	1

(b)

B	1		
C	1	2	
D	2	3	3
	A	B	C

(c)



(d)

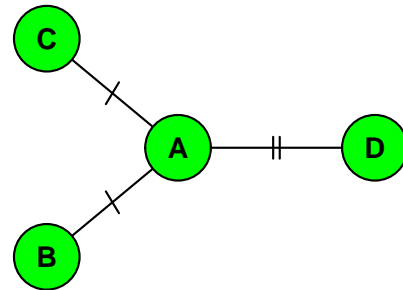


Figure 2: (a) A matrix of four sequences with four sites. (b) The inferred distances. (c) The minimum spanning network (MSN) creates a link between B and C when checking the distances of length two which has the same length than the path B–A–C. (d) The randomized minimum spanning tree (RMST) does not have additional link and is identical to the minimum spanning tree (MST).

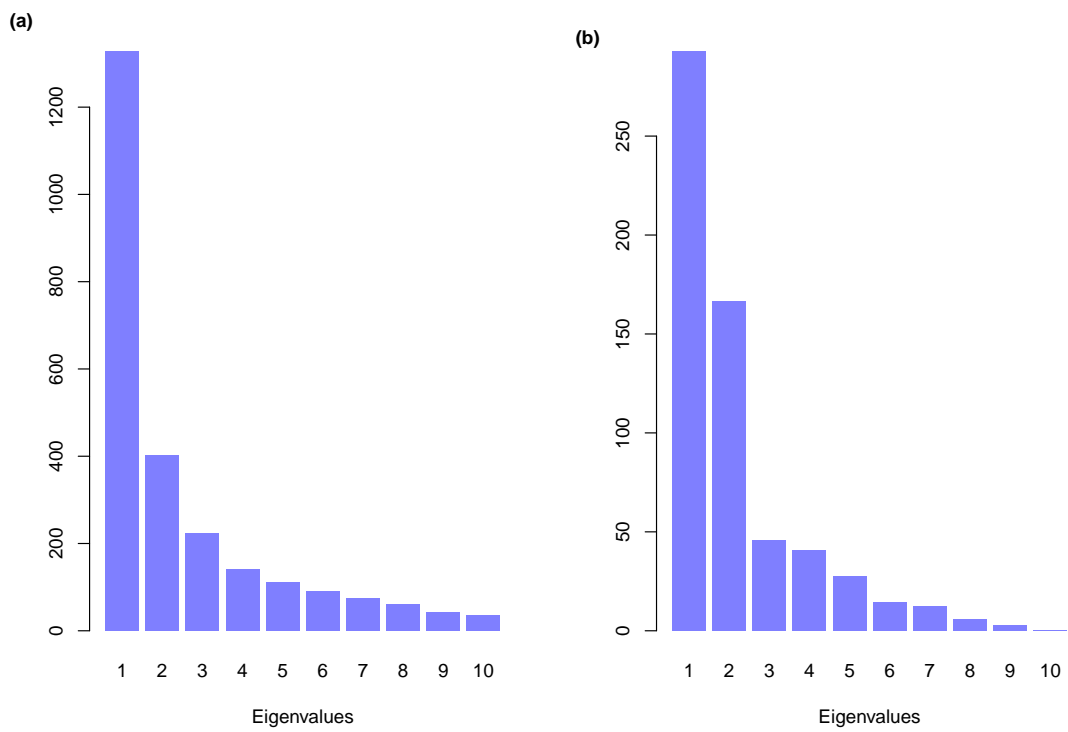


Figure 3: Eigenvalues extracted from the distance matrix for (a) leopards and (b) jaguars.

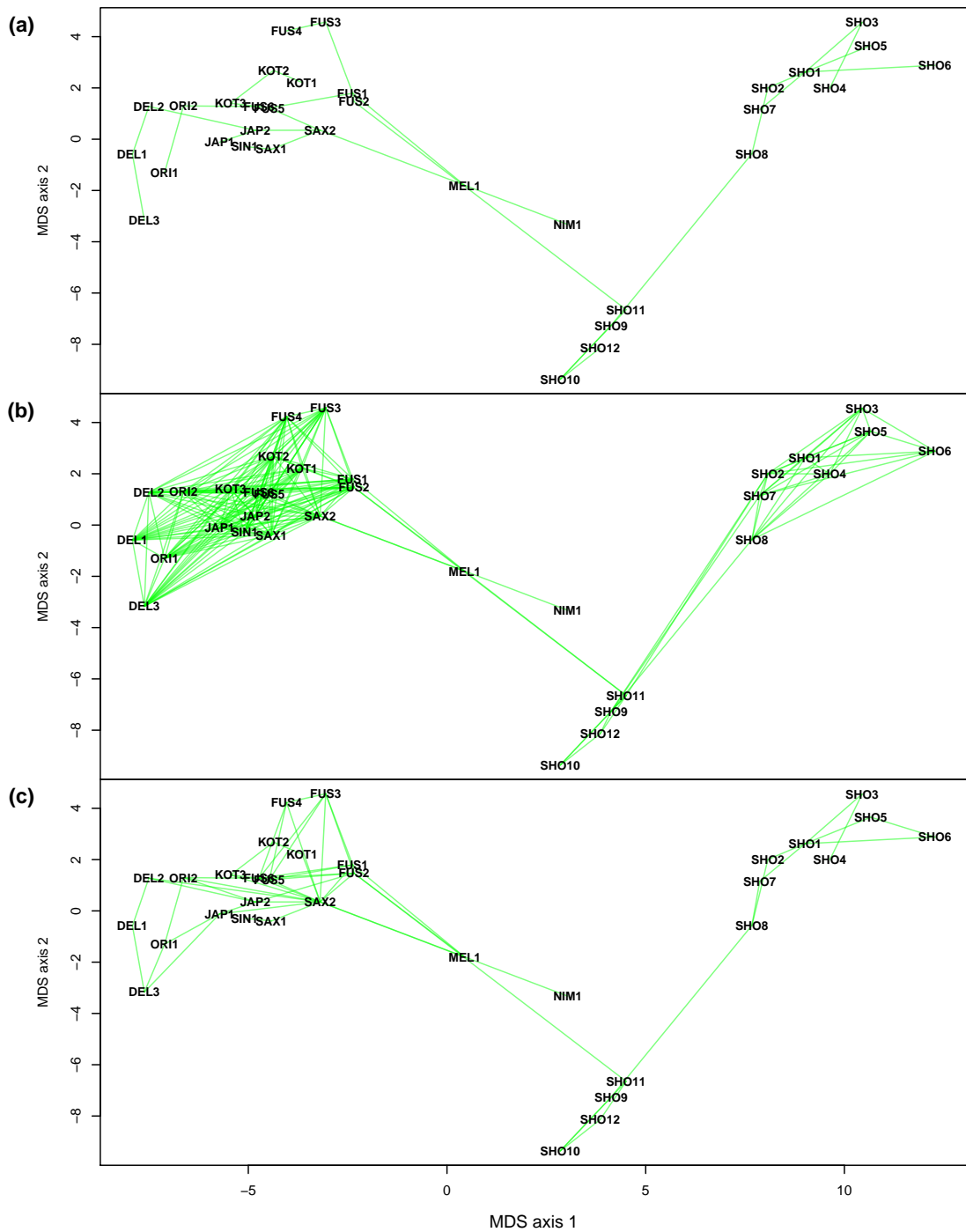


Figure 4: (a) Minimum spanning tree, (b) minimum spanning network, and (c) randomized minimum spanning tree for the leopard data.

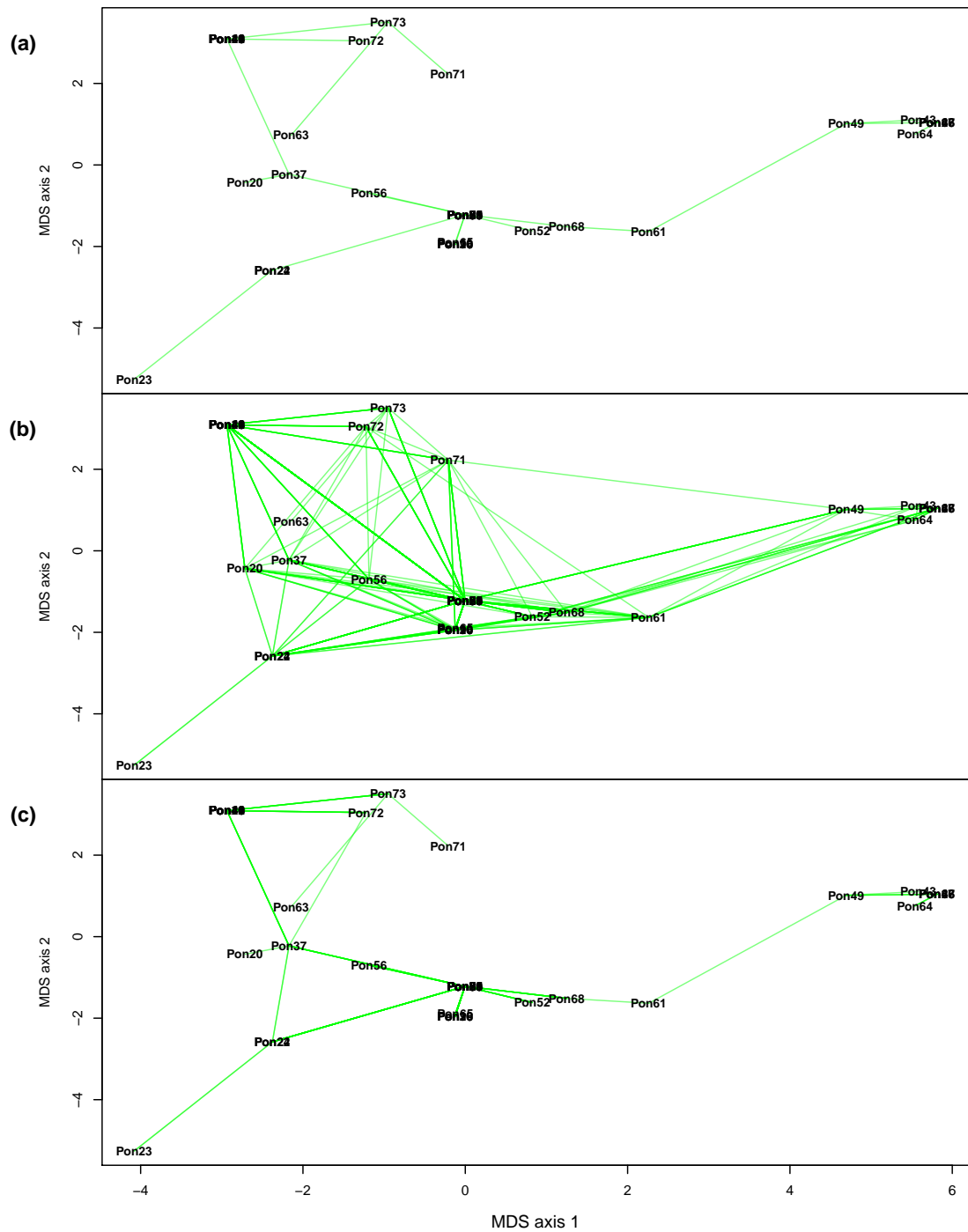


Figure 5: (a) Minimum spanning tree, (b) minimum spanning network, and (c) randomized minimum spanning tree for the jaguar data.