ANeCA: User Guide

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Chapter 1

Software Documentation

This software is a basic fully automated implementation of Nested Clade Phylogeographic Analysis (NCPA). NCPA is a method of phylogeographic inference developed by Alan Templeton and colleagues.

Please become familiar with this manual. It will solve a lot of the common problems encountered using this software. Also please check your results. This software is not intended to be a black box, and is still in early stages of development.

1.0.1 Updates from v1.0

1. The inference key implemented has been updated from 14th July 2004 to the inference key dated 11th November 2005.

Unfortunately I have not had the time to work on including updates of TCS or GeoDis.

1.0.2 Updates from v1.1

1. There has been a change in implementation of Question 19 and 20 on the inference key. These questions will no longer regard populations (sampled or unsampled) within the convex hull of all the clades as within the clade boundary if they are within the convex hull of a subclade.

2. ANeCA now uses TCS v1.21.

3. ANeCA now uses GeoDis v2.5.

1.1 Basic's of NCPA

This is a summary of the method only and my opinion of the state of NCPA. Please familiarise yourself with the literature surrounding the method including criticisms and their replies. If you are unfamiliar with NCPA please use the references within as a guide to familiarise yourself with the method.

1.1.1 Summary of the Method.

Many researchers use NCPA in various ways augmenting the method with various results and techniques. The basic methodology is to generate a haplotype network from your data, nest the haplotype network, calculate statistics, and use an inference key. This is normally applied to a single locus, for example, the Cytochrome b region in mtDNA.

Note that NCPA is a method that has evolved over the years. As such there is some discrepancy as to how NCPA should be applied. The current method of applying NCPA suggested by Templeton et al. (2005) is the following:

1. Generate a haplotype network.

2. Use the nesting algorithm provided in the original literature (Templeton et al. 1987; Templeton and Sing 1993).

3. Calculate the D_n and D_c statistics using GeoDis (Posada et al. 2000).

4. Apply the key either by hand, using AUTOINFER (Zhang et al. 2006)[1], or this software (Check the differences).

5. Validate these inferences using other loci (Templeton et al. 2005).

However in practice many of the articles published by Templeton use the following steps:

1. Generate a haplotype network.

2. Use the criteria in Crandall and Templeton (1993) to find the best resolution of the network.

3. If there are still loops, enumerate all possible trees within the network.

4. Nest all trees (This program is adequate for that purpose).

[1] This software has recently been withdrawn, but may become available in the near future.

5. Calculate the D_n and D_c statistics using GeoDis (Posada et al. 2000).

6. Apply the key either by hand, using AUTOINFER (Zhang et al. 2006), or this software (Check the differences).

7. Check that all the trees provide inferences that are concordant. Discard inferences that do not match.

8. Validate these inferences using other loci (Templeton et al. 2005).

1.1.2 Contraversy of NCPA

The field of phylogeography is currently split as to whether the method works or not. Much of the reasoning behind NCPA is as yet not explained in the literature. Simulations by Knowles and Maddison (2002) indicated that NCPA did not work when they simulated fragmentation. These simulations were criticised by Templeton (2004), however they still provide valuable insights into the performance of NCPA and its usage. Moreover, Templeton (2004) is often considered proof that NCPA is not prone to false positives, and is actually conservative, however it is not the entire story. Much of NCPA is in fact untested. What has been tested is the performance of NCPA under fragmentation, and range expansion, using real data sets with strong a priori expectations on a single loci, using steps 2. and 3. above. NCPA has also been tested using simulations under the scenario of panmixia **without** using steps 2. and 3. on single loci. These simulations shows that NCPA is prone to false positives on a single locus when there is no signal, but remember that this is not the full story. Multi-locus NCPA may still provide the answer to the false positives from multiple testing, but it still needs to be tested.

There is still the question of what effect the criteria of Crandall and Templeton (1993) have on the performance of NCPA. The features used in the criteria are also used later in the calculation of D_c and D_n. How exactly (what is the algorithm) does one say that inferences are concordant? When enumerating all trees in a haplotype network, can a missing intermediate be a tip? If a rooted and unrooted haplotype network both give different inferences in the same clade, is the inference from the unrooted haplotype network false?

1.2 Program citation

This is the citation for this software.
M. Panchal. 2007. The automation of Nested Clade Phylogeographic Anal-

ysis. *Bioinformatics*, **23**:509-510.

Please include the citations for TCS and GeoDis as well, as this software relies heavily upon it.
M. Clement, D. Posada, and K. A. Crandall. 2000. TCS: A computer program to estimate gene genealogies. *Molecular Ecology*, **9(10)**:1657-1659.
D. Posada, K. A. Crandall, A. R. Templeton. 2000. GeoDis: A program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9(4)**:487-488.

1.3 The Input Files

This section gives a description of the input files required to generate the output files. Each file is a plain text file (do not save the files as RTF or DOC files; they are not plain text).

1.3.1 The Nexus or Phylip DNA sequence file

This software uses a modified version of the Nexus file. Labels for each DNA sequence must start with a letter and only contain letters or digits (no whitespace). Each label should also have a period followed by a number indicating which location (see 1.3.2) the sequence came from. Here is an example of a Nexus file.

```
#NEXUS

begin data;
    dimensions ntax=11 nchar=38;
    format datatype=dna missing=? gap=-;
    matrix
mxh1.1      CGTAAAGTTATACCCGAAAGGGAGAGAGTGAGAGTGTG
mxh2.1      CGTAAAGTTTTACCCGAAAGGGAGAGAGTGAGAGTGTG
mxs1.1      CGTAAAGTTATACCCGAAAGGGAGAGAGTGAGAGTGTG
mxs2.1      CGTAAAGTTATAGGCGAAAGGGAGAGAGTGAGAGTGTG
mxs3.2      CGTAAAGTTATAGGGGAAAGGGAGAGAGTGAGAGTGTG
mxs4.2      CGTAAAGTAATAGCCGAAAGGGAGAGAGTGAGAGTGTG
mxs5.2      CGTAAAGTAATAGCCGAAAGGGAGAGAGTGAGAGTGTG
txl1.3      CGTAATGTTATACCCGAAAGGCAGAGAGTGAGAGTGTG
txl2.3      CGTAATGTTATACCCGAAAGGCTCAGAGTGAGAGTGTG
txl3.4      CGTAATGTTATACCCGAAAGGCTCAGAGTGAGAGTGTG
txl4.4      CGTAAAGTTATAGCCGAAAGGCTGAGAGTGAGAGTGTG
```

```
;
end;
```

Note: Although not in the specification of the NEXUS file format, each label and its corresponding DNA sequence should be on a single line (NEXUS sequential). Also the nexus file should not contain any other information other than that which can be seen in the example.

1.3.2 Geographic information file

The first line of the file should contain an identifier for the data set. The second line is a number n that indicates the number of locations sampled (this does not include the unsampled locations specified). The next $2n$ lines contain the entries for each sampled location. Each geographic location specified is written on two lines. The first line of the entry contains the number (id) of the geographic location, followed by the name of the location (letter followed by letters or digits and no whitespace). The second line specifies the sample size, the location in decimal degrees, and the radius (spread) of the sample location (in Km). Further habitable unsampled locations (where prior knowledge indicates the species is present, or there is no prior knowledge about the area) should also be specified after the sampled locations (*This is IMPORTANT information that is used to detect sampling inadequacies*).

This is an example of a geographic information file.

```
mydataset            // Name of the data set
2                    // Number of sampled geographic locations
1 pop1               // Population number and name
7 10.50 -0.23 3      // Sample size, latitude, longitude, radius
2 pop2
5 -5.60 4.50 5.0
3 pop3               // This is an unsampled habitable location
0 -4.50 -3.50 5.4
```

Note: The number for each geographic location should be unique, sequential and start from 1. Furthermore if a geographic region is not included, the assumption made is that the area was sampled and the species was absent in that area, or that the area is uninhabitable for that species.

1.4 The Output Files

This section describes the files that are given as output to the analysis.

1.4.1 The Graph File

TCS automatically writes out a *.graph file when it constructs a haplotype network from the nexus file. The graph file is written in the GML format, and specifies the structure of the haplotype network. It also contains haplotype frequency data and outgroup weights for each sampled haplotype.

1.4.2 The Geodis Input file

This is produced from the *.graph file and the geographic information file. The geodis input file (*.gdin) can be written in two formats. The first format specifies the geographic information as decimal degrees, and is automatically written out. The second format writes the geographic information as a distance matrix where the distances are the great circle distances between each geographic location. The clade information written for both formats are exactly the same.

1.4.3 The GML file

A *.gml file is also written. This contains the structure of the nested cladogram. The file contains a graph in the GML format for each level of the nested cladogram. This file was not intended to be used to construct the nested cladogram design manually. It was intended to be used by the software only. To see what the nested cladogram design looks like please use the *.nest file.

1.4.4 The Nest file

An optional file (*.nest) that can be written so the user can manually reconstruct the nested cladogram design on a diagram of the haplotype network (which can be obtained through TCS). This contains the id of each clade in the nested design. For the 0-step clades, the frequency data is included as well as the label for the clade, and which clades it is connected to. For n-step clades ($n \geq 1$), the information available is the label, the subclades within the clade and the clades that are connected to it. The subclades are specified in the order of tip clades, interior clades, followed by the symmetrically stranded clades.

1.4.5 The NANOVA file

An optional file (*.nanova) can also be written which will provide nested information for each individual in the analysis. The data is provided as partial data (as Comma Separated Values) that can be combined with individuals' phenotype data for analysis with a Nested Analysis of Variance. The file is primarily produced for users that wish to perform genotype-phenotype studies rather than geographic association studies of a species. See 1.7 for more information.

1.4.6 The GeoDis Output file

This file (*.gdout) is written by GeoDis and contains the D_c and D_n probabilities for each clade.

1.4.7 The GeoDis Summary file

This file can be produced by the automated inference key. The input files required for this and the inference file are the geographic information file, the Geodis Input (decimal degrees format), the GeoDis Output file, and the GML file. The GeoDis input file needs to be in the decimal degrees format even if the distance matrix format was analysed by GeoDis. The file contains a summary of the information contained in the GeoDis Output file and marks statistically significant values with a star (and s or l if it is statistically small or statistically large). It also contains geographic locations and their spread. The geographic distribution is also given for each clade.

1.4.8 The Inference File

This file contains the inference made for each clade and the chain of inference showing which questions were answered to reach that conclusion.

1.5 Using the software for NCPA

This is a basic guide to using the software for geographic association studies of a species.

1.5.1 Using the Graphical Interface

To run the software either select `NCPA.jar` in your file explorer or type

```
java -jar NCPA.jar
```

from the command line.

To create the haplotype network using TCS v1.18, select

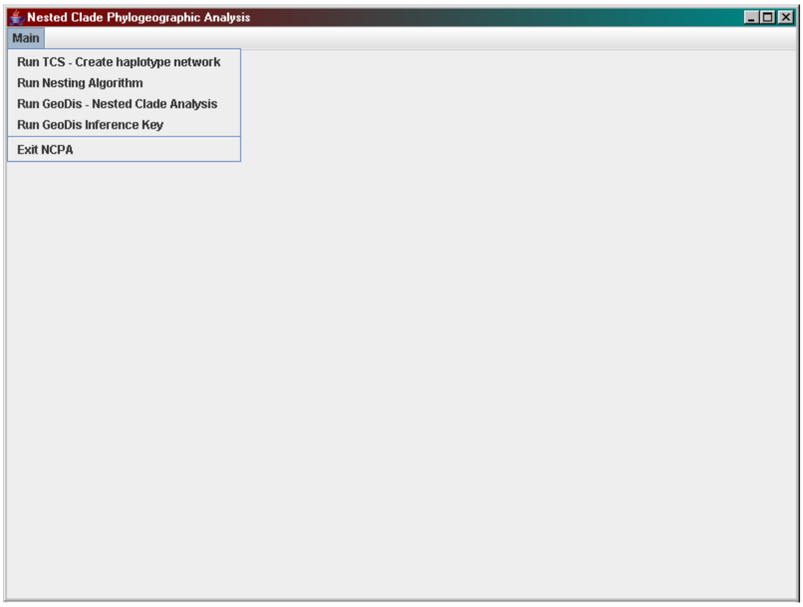

Nested Clade Phylogeographic Analysis
Main
Run TCS - Create haplotype network
Run Nesting Algorithm
Run GeoDis - Nested Clade Analysis
Run GeoDis Inference Key
Exit NCPA

Figure 1.1: How to start TCS.

```
Main > Run TCS - Create haplotype network
```

from the menu.

Then select your nexus file from the menu using the following command,

```
File > Select NEXUS/PHYLIP Sequence file
```

and then click `RUN`, which will automatically save a *.graph file. Once the haplotype network is created it is a good idea to save a picture of the haplotype network as well, although this can also be done at a later stage. Click

```
File > Save network as postscript
```

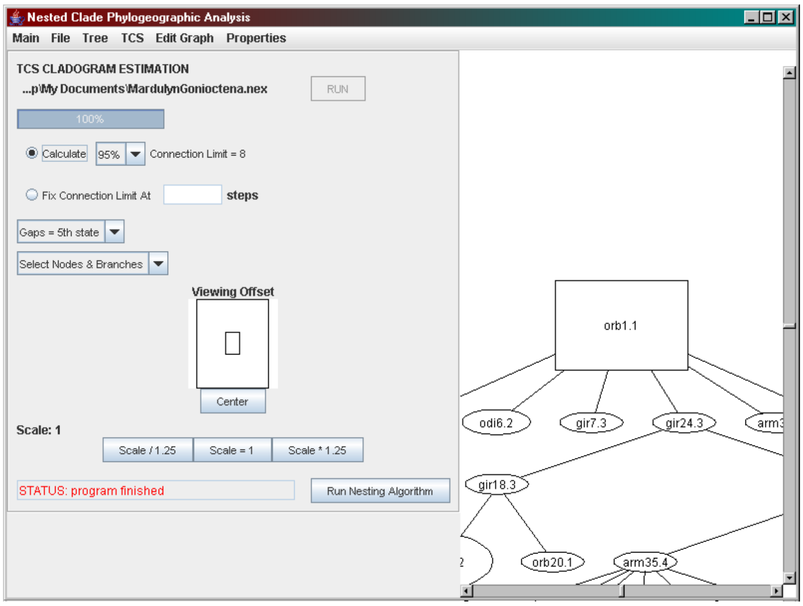
to save the image as a postscript file. Please refer to the TCS documentation for further information about its functionality.

The next step is to nest the haplotype network and is done by either selecting

```
Main > Run Nesting Algorithm
```

from the menu or by clicking the `Run Nesting Algorithm` button.

The following interface is displayed, and if TCS was used before, the textfields should be automatically completed providing the files can be found. The user must provide the locations of the TCS graph file and the file con-



TCS CLADOGRAM ESTIMATION

...p\My Documents\MardulynGonioctena.nex

100%

● Calculate 95% Connection Limit = 8

○ Fix Connection Limit At steps

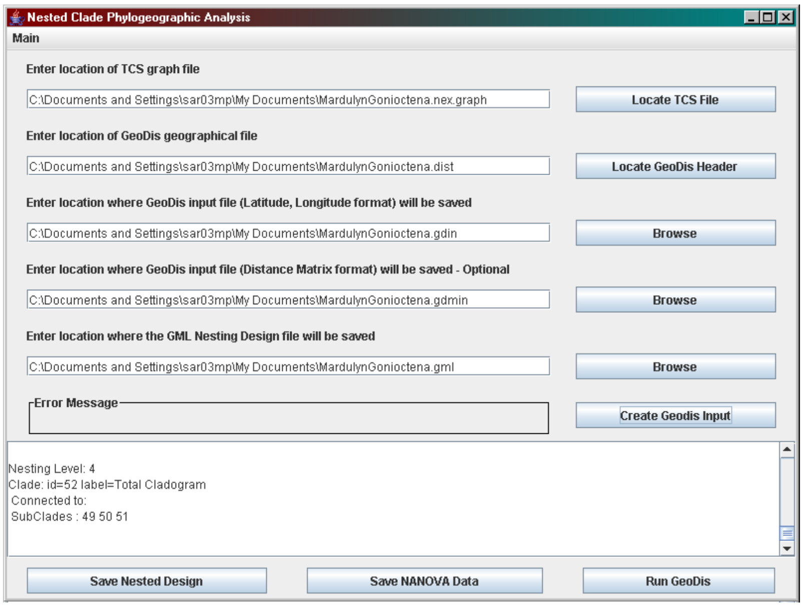
Gaps = 5th state

Select Nodes & Branches

Scale: 1

STATUS: program finished

Figure 1.2: A screen shot of TCS.



Enter location of TCS graph file

C:\Documents and Settings\sar03mp\My Documents\MardulynGonioctena.nex.graph

Enter location of GeoDis geographical file

C:\Documents and Settings\sar03mp\My Documents\MardulynGonioctena.dist

Enter location where GeoDis input file (Latitude, Longitude format) will be saved

C:\Documents and Settings\sar03mp\My Documents\MardulynGonioctena.gdin

Enter location where GeoDis input file (Distance Matrix format) will be saved - Optional

C:\Documents and Settings\sar03mp\My Documents\MardulynGonioctena.gdmin

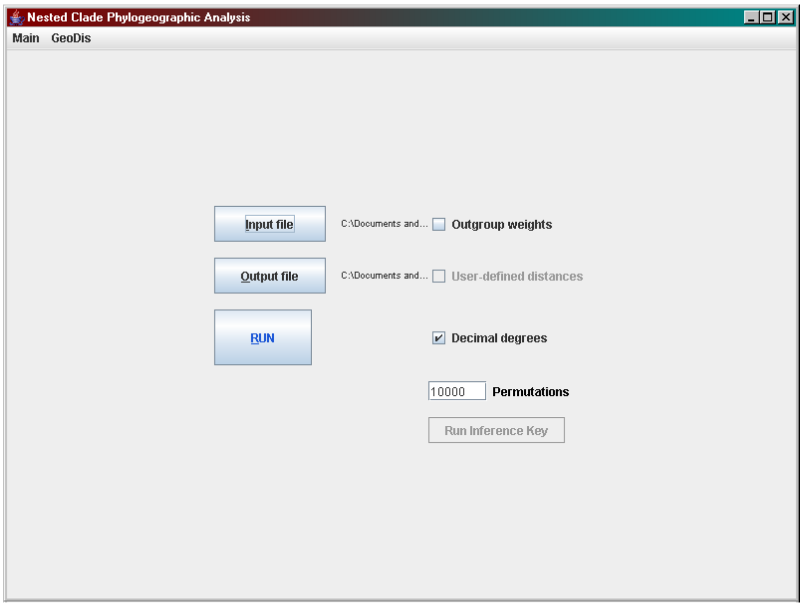
Enter location where the GML Nesting Design file will be saved

C:\Documents and Settings\sar03mp\My Documents\MardulynGonioctena.gml

Error Message

```
Nesting Level: 4
Clade: id=52 label=Total Cladogram
 Connected to:
 SubClades : 49 50 51
```

Figure 1.3: A screen shot of the nesting software.



C:\Documents and... □ Outgroup weights

C:\Documents and... □ User-defined distances

☑ Decimal degrees

10000 Permutations

Figure 1.4: A screen shot of GeoDis.

taining the geographic information. The names of the GeoDis input files and the name of the GML nested design file also need to be specified as they will be required later on. Although the Distance Matrix format form of the GeoDis file is optional it might be a good idea to compare the analyses from both forms of input.

By clicking the `Create GeoDis Input` button, a summary of the nested design will be shown in the text area that can be used to reconstruct it on the haplotype network. This can also be saved by clicking `Save Nested Design`.

To move on to GeoDis select,

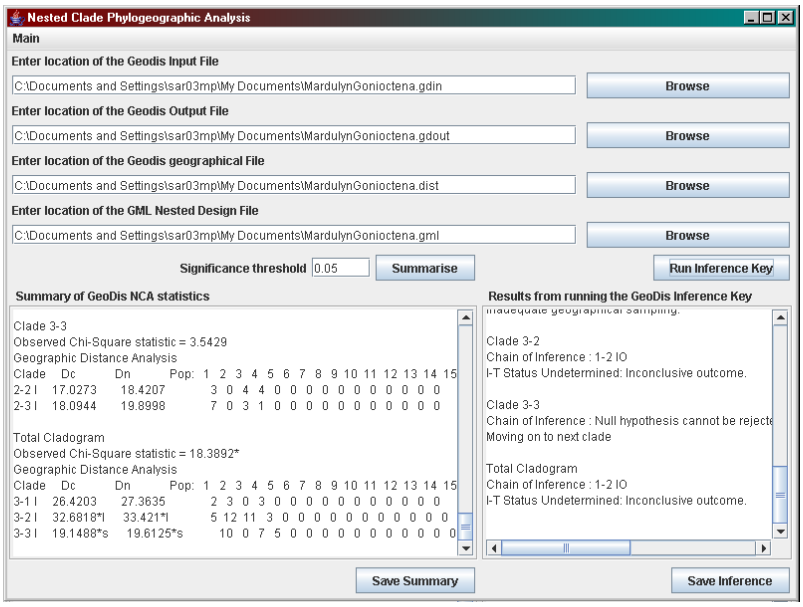
```
Main > Run GeoDis - Nested Clade Analysis
```

from the menu or click on the button `Run GeoDis`, which displays the GeoDis interface.

If the nesting algorithm has just been run, the default input and output files specified will be for the latitude-longitude format. Select the `Decimal degrees` checkbox and then click `RUN`. If you want to analyse the distance matrix format, then the input and output files need to be reselected. Following that select the `user-defined distances` checkbox and then click `RUN`.

The automated inference key is then run by selecting

```
Main > Run GeoDis Inference Key
```



Enter location of the Geodis Input File: C:\Documents and Settings\sar03mp\My Documents\MardulynGonioctena.gdin

Enter location of the Geodis Output File: C:\Documents and Settings\sar03mp\My Documents\MardulynGonioctena.gdout

Enter location of the Geodis geographical File: C:\Documents and Settings\sar03mp\My Documents\MardulynGonioctena.dist

Enter location of the GML Nested Design File: C:\Documents and Settings\sar03mp\My Documents\MardulynGonioctena.gml

Significance threshold: 0.05

Summary of GeoDis NCA statistics

```
Clade 3-3
Observed Chi-Square statistic = 3.5429
Geographic Distance Analysis
Clade   Dc         Dn          Pop:  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15
2-2 I   17.0273    18.4207           3  0  4  4  0  0  0  0  0  0  0  0  0  0  0
2-3 I   18.0944    19.8998           7  0  3  1  0  0  0  0  0  0  0  0  0  0  0

Total Cladogram
Observed Chi-Square statistic = 18.3892*
Geographic Distance Analysis
Clade   Dc         Dn          Pop:  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15
3-1 I   26.4203    27.3635           2  3  0  3  0  0  0  0  0  0  0  0  0  0  0
3-2 I   32.6818*l  33.421*l          5 12 11  3  0  0  0  0  0  0  0  0  0  0  0
3-3 I   19.1488*s  19.6125*s          10  0  7  5  0  0  0  0  0  0  0  0  0  0  0
```

Results from running the GeoDis Inference Key

```
inadequate geographical sampling.

Clade 3-2
Chain of Inference : 1-2 IO
I-T Status Undetermined: Inconclusive outcome.

Clade 3-3
Chain of Inference : Null hypothesis cannot be rejecte
Moving on to next clade

Total Cladogram
Chain of Inference : 1-2 IO
I-T Status Undetermined: Inconclusive outcome.
```

Figure 1.5: A screen shot of the automated inference key.

from the menu or clicking on the `Run Inference Key` button. Again the textfields are automatically filled in if GeoDis was used before. The files required are the GeoDis input file in latitude-longitude format (even if you are analysing the distance matrix format output from GeoDis), the GeoDis output (in either latitude-longitude or distance matrix format; the default is latitude-longitude format), the geographic information and the GML nested design. By clicking `Summarise`, a summary of the geodis statistics are created. The summary contains the location of the sample sites and the clades analysed. For each clade analysed the summary marks which distances are significant at the 5% level with a *s or a *l (significantly small or large respectively), and also the sample location distribution. Click `Run inference key` to run the automated inference key. This will analyse each clade and give the chain of inference and the final outcome, but nothing more. To find out which locations were involved in which inferences, the automated inference key must be traced manually using the summary file and the chain of inference.

Note: GeoDis and the automated inference key may take a long time to run for large data sets. Please be patient (To be sure an error has not occured it may be better to run the graphical interface from the command line).

1.5.2 Using the Command Line

By using the command line all the files described above are written to disk with the same name stem as the DNA sequence file (*i.e.*, the NEXUS or PHYLIP file), including the analyses with the distance matrix form (these are tagged with an m before the file ending *e.g.*, filename.minfer or filename.mgdin). To execute the software, type on the command line,

```
java -jar NCPA.jar [dnaSeqFile] [geographicfile]
```

e.g.,

```
java -jar NCPA.jar mydataset.nex mydataset.dist
```

Currently there is no provision to run individual components of the automated software, and so if you want to conduct a portion of the analysis, it must be done from the graphical interface (or you can modify the code).

Additional options are available that must be specified in the given order. These are the number of permutations that GeoDis uses (default is 10,000), whether to use the distance matrix format (0 - false, 1 - true), and the significance threshold to use (default is 0.05). Again this is run on the command line as follows.

```
java -jar NCPA.jar [dnaSeqFile] [geographicfile]
            [numberOfPermutations] 0 [significanceThreshold]
```

All five parameters must be included even if you only wish to change one of them.

1.5.3 Drawing the nested design

When the nesting software has finished writing the GeoDis Input file, you have the option of saving a summary of the nested design that can be used to draw it out. The first step is to print out a picture of the haplotype tree from TCS. If you have disjoint networks present TCS will only draw out one of them, but by using the options from the menu the other networks can be drawn out separately as well.

The summary of the nesting design contains all the levels of nesting starting from the haplotype network. Each clade, including missing intermediates, have a corresponding id with which to identify them. By using the labels of the clades, haplotypes on the picture of the network can be assigned their ids. Each clade also indicates which clade it was connected to allowing missing intermediates to be assigned their ids. 1-step and higher level clades also specify the ids of their subclades, and so the nesting design can be gradually built up. The subclade ids are specified in the order of tip clades, interior clades, and symmetrically stranded clades, which is helpful to determine how the network was nested.

1.6 A Worked example

The files in this example are provided in the directory WorkedExample. The data set provided is a real data set from (Mardulyn 2001). This example uses the decimal degrees notation, however it is still possible to analyse distances in GeoDis using the distance matrix format. I explain how this is done at the appropriate point in the example.

1.6.1 Creating the Geographic information file

Here is a step by step guide to creating the Geographic information file.

1. Type the name of the dataset on the first line of the file.

2. On the second line of the file type the number of populations sampled.

3. On the following lines we then type the information for each sampled area. The information for each sampled area takes 2 lines. On the first is the population identification number (starting at 1) followed by the name of the area (at least 3 characters long, consisting of letters and numbers and no white space). The second line of the information contains the sample size, latitude (in decimal degrees), longitude (in decimal degrees), and the radius (in Km) of the sample size.

4. After the sampled areas are the unsampled habitable areas. These are locations where the species is known to be but was unsampled (e.g. inaccessable areas), or where the area is habitable but the presence of the species is unknown. This information takes 2 lines for each area. The first line is the population identification number (continuing numbering from the last sampled population), followed by the location name. The second line is the sample size (which is 0), the latitude (decimal degrees), the longitude (decimal degrees), and the area radius (Km).

5. Save the file as a plain text file.

See `GonioctenaPallida.dist` for the example file.

1.6.2 Creating the Nexus file

This is a step by step guide to creating the Nexus file in sequential NEXUS format.

1. On the first line of the file type `#NEXUS` and then leave a line.
2. Type `begin data;`.
3. Following this type `dimensions ntax=` followed by the number of individuals in your data set. On the same line type `nchar=` followed by the length (number of bases) of the DNA sequences and a semicolon. (including insertions and missing characters represented by a `-` and `?` respectively).
4. On the next line type `format datatype=dna missing=? gap=-;`.
5. Then `matrix` on the next line.
6. On the next `ntax` lines enter a label for each of your dna sequences. Ensure the labels start with a letter and contain only letters or digits (no whitespace or special characters).
7. At the end of each label type a period followed by the identification number of the population/deme it came from.
8. Follow each label with a space or tab followed by the DNA sequence.
9. Once all the DNA sequences are written, on a new line type `;`.
10. On the new line type `end;`.
11. Then save the file as a plain text file with the file ending `.nex`.

See `GonioctenaPallida.nex` for the example file.

1.6.3 Create the haplotype network

Open ANeCA, and select `Main > Run TCS` (or the latest version of TCS). Open the Nexus file and click `Run`. At this stage it is your choice how you wish to resolve loops, if they are present. In this example we follow the steps of Mardulyn (2001) and resolve loops using two criteria suggested by Crandall and Templeton (1993): (i) rare haplotypes are more likely to be found at the tip, and more common haplotypes at interior nodes, of a cladogram; and (ii) a singleton is more likely to be connected to haplotypes from the same population than to haplotypes from different populations (However, note warning above about resolving loops). Once we have identified which edges/branches and missing intermediate haplotypes need to be removed, we need to identify the identification numbers of the missing intermediate haplotypes and the haplotypes which the branches connect to in order to modify the *.graph file. To do this, visually locate the nodes and edges which need to be removed. By double-clicking on a node that needs to be removed or is connected to edge that needs to be removed, a dialog box will popup. In the title bar will be the words Node x, where x is the identification number of the node. For each node that needs to be removed, record this number. For each edge that needs to be removed, record both nodes the edge is connected to. Open the graph file (in this case GonioctenaPallida.nex.graph) written by TCS in a plain text editor. Using the search function, search for the string `id` x where x is the identification number of the node to be removed. This string (`id` x) will be nested in a `node [ ...]` block. Delete this block to remove the node. For each edge to be removed, search for the string `source` x. This will identify `edge [ ...]` blocks where one of the haplotypes is connected to the edge to be removed. Check that the `target` y corresponds to the other haplotype the edge is connected to. If this is true, then remove that `edge [ ...]` block. Repeat this, searching for `target` x and checking the `source` y, to ensure the edges that need to be removed have been. Save this file as plain text and then continue with the nesting. Note that although it is possible to modify visually the haplotype network within TCS, this cannot be saved as a graph file. However please take the opportunity to save a picture of the haplotype network.

1.6.4 Creating the Nested Design

Click `Run Nesting Algorithm` from TCS or from the menu. Select the graph file and the geographic information file to be used in nesting. Select the location to save the GeoDis input file (GonioctenaPallida.gdin) and the GML nested design (GonioctenaPallida.gml), and then click `Create GeoDis Input`. In order to draw and check the nested design you will need to save the summary of the nested design (GonoictenaPallida.nest), by clicking `Save Nested Design`. If you wish to write out the GeoDis input with geographic information as a distance matrix then specify the name of that file too (GonioctenaPallida.gdmin).

1.6.5 Drawing the Nested Design

Print out a picture of the haplotype network, and open the nest file (GonioctenaPallida.nest) in a plain text editor. At the start of the file will be a count of the number of edges in the graph and a count of how many are part of loops. Next is the clade information at `Nesting Level: 0`, the haplotype network level. Each `Clade:` gives the identification number of the haplotype/clade and the label it has on the diagram of the haplotype network. Clades with `label=" "` indicate the clade is a missing intermediate haplotype. On the diagram of the haplotype network label each node with the id of the clade, including missing intermediate haplotypes. Labeling missing intermediate clades may be difficult, however each `Clade:` shows the id's of the clades it is connected to. Using this information it is possible to deduce which id's belong to which missing intermediate clades. When all the haplotypes on the diagram have been labeled, move onto `Nesting Level: 1`. For each `Clade:` in this level use `SubClades :` identify which group of haplotypes/clades have been nested, and draw the clade onto the diagram. Mark the clade id next to each clade drawn onto the diagram. Continue this for each nesting level, checking that clades are connected to the correct clades.

1.6.6 Performing the Permutation Analysis

Click `Run GeoDis`, and select the GeoDis input file to be analysed (either GonioctenaPallida.gdin or GonioctenaPallida.gdmin), and select either `Decimal degrees` or `User-defined distances` depending on the input file chosen. Both input files have been analysed here (GonioctenaPallida.gdout corresponds to the input file GonioctenaPallida.gdin, and GonioctenaPallida.gdmout corresponds to the input file GonioctenaPallida.gdmin).

1.6.7 Applying the Automated Inference Key.

Click `Run Inference Key` to open the automated inference key. Select the GeoDis input file in decimal degree format (GonioctenaPallida.gdin), the GeoDis output file from either format (GonioctenaPallida.gdout or GonioctenaPallida.gdmout) depending on the statistics you wish to analyse, the geographical information file (GonioctenaPallida.dist), and the GML file (GonioctenaPallida.gml). Then click `Summarise` and `Run Inference Key`. This will summarise the information required to apply the key, and present the inferences made for each clade using the automated key. To check each inference (GonioctenaPallida.infer for the analysis using decimal degrees, and

GonioctenaPallida.minfer for the analysis using the user-defined distances) follow the inference key using the summary file (GonioctenaPallida.gdsum for the analysis using decimal degrees format, GonioctenaPallida.gdmsum for the analysis using the user-defined distances). For each clade the summary file contains the D_c and D_n values and their significance, and also the number of individuals at each location. This allows the user to mark out a clades geographic boundaries on a map and see which individuals and locations are involved in an inference.

1.7 Using the software for genotype-phenotype studies

Nested Clade Analysis was originally designed for genotype-phenotype association studies (although this software was initially designed only for NCPA). A short guide is provided for using the software for genotype-phenotype association studies.

Note: NCA for genotype-phenotype association studies has been superceded by TreeScan (Posada et al. 2006), in particular to address the multiple testing problem.

1.7.1 File simplifications

Although some users may also want to look at geographic associations, the data specified for NCPA is more than is required for genotype-phenotype association studies. If the user is not concerned about geographic location then the distance information file need only contain the following few lines.

```
data_set_name
1
1 pop_1
1 0.0 0.0 0
```

The DNA sequence file normally would include the geographic location in the label of each individual, however this should be replaced by the index 1 (corresponding to the dummy location in the geographic information file).

```
#NEXUS

begin data;
    dimensions ntax=11 nchar=38;
    format datatype=dna missing=? gap=-;
    matrix
mxh1.1      CGTAAAGTTATACCCGAAAGGGAGAGAGTGAGAGTGTG
mxh2.1      CGTAAAGTTTTACCCGAAAGGGAGAGAGTGAGAGTGTG
mxs1.1      CGTAAAGTTATACCCGAAAGGGAGACAGTGAGTGTGTG
mxs2.1      CGTAAAGTTATAGGCGAAAGGGAGACAGTGAGAGTGTG
mxs3.1      CGTAAAGTTTTAGGGGAAAGGGAGACAGTGAGAGTGTG
mxs4.1      CGTAAAGTAATAGCCGAAAGGGAGAGAGTGAGAGTGTG
mxs5.1      CGTAAAGTAATAGCCGAAAGGGAGAGAGTGAGAGTGTG
```

1.7.2 Using the Graphical Interface

TCS and the automated nesting algorithm is used in the same way as for NCPA. The only difference is that an extra file needs to be saved. Once the nesting is complete, Click on `Save NANOVA Data`. This saves the data for each individual, row by row specifying which clade the individual is in at each step of the nested design. Note that TCS does not necessarily produce a haplotype tree, and therefore the graph file will need to be modified before nesting is performed. Often techniques from Coalescent Theory are used to resolve loops (see Crandall and Templeton (1993) and Pfenninger and Posada (2002)), or all possible trees are explored.

1.7.3 Using the Command line

To run NCA from the command line type:

```
java -jar NCPA.jar [dnaSeqFile] [geographicfile] -NANOVA
```

This causes the software to omit the GeoDis and automated inference key stages of a normal NCPA run.

1.8 Limitations of Software

Careful consideration needs to be given to regions crossing the +180/-180 line of longitude. For smaller areas of coverage all the geographic co-ordinates can be offset such that the area does not lie over the line. This assumes the species cannot migrate in both directions around the globe to reach locations either side of the +180/-180 line of longitude.

The automated inference key uses spherical geometry to some extent and great circle distances between locations, and so is unsuitable for use in situations in which the distance matrix format in GeoDis would traditionally be used (*e.g.*, for riparian species).

Lastly, if NCPA appears not to respond then it is likely that an exception has been thrown. Please run the application from the command line using `java -jar NCPA.jar`. Then duplicate the problem and note the full output from the command line (redirect the output to a file is you area able to using `java -jar NCPA.jar > output.txt`). Then send this to m.panchal@rdg.ac.uk explaining how the bug occurred, how to replicate it, the input files, and the output from the command line.

Note: If the Exception thrown is an OutOfMemoryException you can try the following solution. Type the following on the command line.

```
java -Xms256m -Xmx256m -jar NCPA.jar ([additional arguments])
```

Lastly please consult the developers documentation for further information regarding the software, such as the design and implementation details.

1.9 Common Exceptions thrown

We have attempted to make this software as easy to use as possible however you may encounter certain exceptions while running ANeCA. Most exceptions generally note the problem and not the cause. We try to explain the cause here and how they can be corrected.

OutOfMemoryException This exception indicates that the Java Virtual Machine (JVM) has used up the memory allocated to this process. To correct this the amount of memory the JVM uses must be increased, using the flags -Xms and -Xmx. Type the following on the command line.

```
java -Xms256m -Xmx256m -jar NCPA.jar ([additional arguments])
```

This exception most often occurs in large datasets.

NumberFormatException:"unable to parse number" This indicates that the geographic information file has been incorrectly written. Please check this against the examples given.

1.10 Known Issues

Random exceptions thrown for Mac users Various exceptions have been thrown running this software on Mac Operating Systems. Often the exception cannot be reliably replicated and therefore the source of these seemingly random exceptions is unknown.

Rerunning GeoDis After GeoDis has been run once, under certain circumstances it does not run again unless the software is restarted. The circumstances are as yet unknown.

1.11 Frequently Asked Questions

How do I specify the radius of a sample location?
The radius of a sample location in effect describes the approximate movement range of individuals in that location, and also how far the sampled individuals were spread out. For example, plants may be sampled from a large area, or insects may be caught using a single trap but will have traveled to it within a general radius. This parameter is slightly subjective, however it means that the sample location covers a certain area rather than a point location.

How do I specify the unsampled locations?
This is done in the same way as for the sampled locations. These locations are important because you either don't know if something is there or you know something is there but haven't been able to sample it. Here the radius can be used to describe the approximate size of the unknown area. It is up to the user to determine how many of these location need to be specified and to what granularity.

Why should I include unsampled locations?
If no unsampled locations are included, the software assumes that the species is absent in that area. This also means the safeguards introduced in the inference key to guard against sampling inadequacies are bypassed.

My study area is too small/large to specify the radius in Km. What do I do?
NCPA is insensitive to scale, and so geographic co-ordinates can be safely scaled, translated, and rotated to make radius distances in Km more sensible.

I want to resolve loops. How can I do this?
This software currently offers no option of resolving loops. It is still possible to resolve them though and continue to use the software. First run the nesting software on the reticulate network and save the *.nest file. Print a picture of the haplotype network, and then use the *.nest file to assign the ids to the haplotypes. Use your favoured method of breaking/resolving loops to determine which edges should be removed. Open up a plain text editor and open up the *.graph file automatically written by TCS (should be in the

same directory as your nexus file). In front of you now will be what looks nonsensical information. Scroll down until you reach lines that looks like this.

```
edge [
    source 3
    target 5
]
```

This is a representation of an edge. The numbers after `source` and `target` are the ids of the haplotypes. Find the edges that you want to remove and delete those four lines corresponding to each edge. For example if there is an edge that links haplotype with id 4 to a haplotype with id 7 then the code will look either like this,

```
edge [
    source 4
    target 7
]
```

or like this.

```
edge [
    source 7
    target 4
]
```

By removing these lines you have removed the edge connecting haplotype 4 to haplotype 7. Make sure you save this file as a plain text file. Open up the nesting software and specify the file you just saved as the TCS graph file. You can then continue using the software and nest the modified haplotype network.

I have external information that makes some of the clades interior clades rather than tip clades. How do I change this in the program?

Changing the tip/interior status of a clade is not currently possible within the program. Once the GeoDis input file has been written, it can be changed manually in a plain text editor. See the GeoDis documentation for further information regarding the GeoDis input specification. Once editing of the files is complete, it can be read into GeoDis.

I want to use another haplotype network estimation method instead of Statistical Parsimony. How do I use it with this software?
The software currently nests haplotype networks that have been written in the GML format that is written as standard by the TCS software. To use a haplotype network that is produced by another haplotype network estimation method, it must first be converted/written in the GML format. Unfortunately there is no software that currently does this, and so the graph file must be written by hand.

I have found a bug in the software. Who do I contact?
Please check if it is a bug, and there is no solution mentioned in the manuals (either this or the TCS or GeoDis manuals). If it is a problem regarding the correctness of TCS output or GeoDis output, please use the latest version of the program available on David Posada's website. If the problem persists, please contact David Posada. If the problem is to do with the graphical interface not functioning correctly (in TCS or GeoDis as well), or regarding the nesting software or the automated inference key, please contact me.

A newer version of TCS/GeoDis is available. Why is it not included?
I haven't had the time to incorporate the new versions yet.

The software has stopped responding. What happened?
If it is a large data set please be patient with the software. If you have made modifications to the input files then it is possible that there may be an error reading the files. If you think a problem has occured, close the software, and run it again from the command line using `java -jar NCPA.jar` from where the NCPA.jar file is located.

1.12 Disclaimer

This software is provided free of charge. This program is distributed in the hope that it will be useful, but without any warranty; without even the implied warranty of merchantability or fitness for a particular purpose. The utmost effort has been made to ensure the program is correct and free from bugs, however problems may arise.

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