**Supp. file 1.** Additional information. <https://doi.org/10.5852/ejt.2022.852.2003.8181>

Table S1. PCR primers used in this study along with their sequences.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Gene abbreviation** | **Forward/Reverse** | **Primer name** | **Sequence (5’ to 3’)** | **Reference** |
| Cyt b | F | Gludgmod | CTT GAA AAA CCA CCG TTG T | Dawson et al., 2008 |
| Cyt b | R | H16064mod | GGT TTA CAA GAA CAA YGC T | Dawson et al., 2008 |
| ND4 | F | ND4 | CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC | Arevalo et al., 1994 |
| ND4 | R | LEU | CAT TAC TTT TAC TTG GAT TTG CAC CA | Arevalo et al., 1994 |
| 12S | F | L1091 | AAA CTG GGA TTA GAT ACC CCA CTA T | Knight and Mindell, 1993 |
| 12S | R | H1557 | GTA CAC TTA CCT TGT TAC GAC TT | Knight and Mindell, 1993 |
| 16S | F | L2510 | CGC CTG TTT ATC AAA AAC AT | Palumbi, 1996 |
| 16S | R | H3059 | CCG GTC TGA ACT CAG AT | Palumbi, 1996 |
| CMOS | F | S77 | CAT GGA CTG GGA TCA GTT ATG | Lawson et al., 2005 |
| CMOS | R | S78 | CCT TGG GTG TGA TTT TCT CAC CT | Lawson et al., 2005 |
| PRLR | F | PRLR-NEW\_F | CCR GAT TCT GAT GAG RTT CAA AGG AAC | Max Jones, MZool thesis, unpublished |
| PRLR | R | PRLR-NEW\_R | CTG GAG GCA CTA ACC AGA ATG CCT CTT G | Max Jones, MZool thesis, unpublished |
| UBN1 | F | BaUBN\_F | CCT CTG GTT ACT CAG CAG CA | Casewell et al., 2011 |
| UBN1 | R | BaUBN\_R | ATT GGT CAC TCC TTG TGT TC | Casewell et al., 2011 |
| RAG1 | F | R13 | TCT GAA TGG AAA TTC AAG CTG TT | Groth et al., 1999 |
| RAG1 | R | R18 | GAT GCT GCC TCG GTC GGC CAC CTT T | Groth et al., 1999 |
| NT3 | F | NTF\_SC\_F | GCA TTT CTG TGT GGC ATC CA | Sonia Cremer PhD thesis, unpublished |
| NT3 | R | NTF\_SC\_R | CGA GGT TTT GCA CTG GGA AT | Sonia Cremer PhD thesis, unpublished |

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Table S2. The PCR programs used for each gene in this study. The range given for the annealing temperatures indicates that some variation was required to produce usable PCR products.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Gene** | **Initial Denaturation** | **Denaturation** | **Annealing** | **Extension** | **No. of cycles** | **Final Extension** |
| **Cyt b** | 94°C, 2min | 94°C, 30sec | 55°C, 30sec  (55°C - 59°C) | 72°C, 1min | 40 | 72°C, 5 min |
| **ND4** | 57°C, 30sec | 72°C, 1min | 35 |
| **12S** | 45°C, 30sec | 72°C, 45sec | 35 |
| **16S** | 50°C, 30sec | 72°C, 45sec | 35 |
| **CMOS** | 56°C, 30sec  (56°C - 62°C) | 72°C, 1min | 35 |
| **PRLR** | 50°C, 30sec | 72°C, 30sec | 40 |
| **UBN1** | 60°C, 30sec  (55°C - 60°C) | 72°C, 45sec | 40 |
| **RAG1** | 54°C, 45sec  (48°C - 54°C) | 72°C, 1min | 40 |
| **NT3** | 58.5°C, 30sec  (58°C - 64°C) | 72°C, 1min | 35 |

**RWTY diagnostics and interpretation**

Figure S1. Parameter Trace Plots- RWTY will produce trace and density plots of each column statistic in the trace file. For example, in this study, a parameter trace and density plot were created for the log likelihood of each cold chain (LnL). Such plots are useful to help tune further analysis (e.g., burn-in). The title of each plot shows the Effective Sample Size (ESS). In general, all the parameters should have an ESS>200, which was achieved in this study.

Timeline

Description automatically generatedTimeline

Description automatically generated

Figure S2. Tree topology plots are like the parameter plots. They reveal where the MCMC was sampling from a stationary distribution of chains or whether moving between different modes. They also show how well-mixed the chains are with respect to tree topologies. Well-mixed chains will show the trace jumping rapidly between values.

Timeline

Description automatically generatedTimeline

Description automatically generated

Figure S3. The plots show the split frequencies of clades in the chain either as the frequency calculated from cumulative samples in the chain at various points in the analysis or as the frequency within the sliding window. By default, they show the 20 clades with the highest standard deviation od split frequencies within each sample. In analysis that is mixing well, the frequencies in the cumulative plot should level off and the frequencies in the sliding window to move around a lot. If the frequencies in the cumulative window are not levelled off, it may mean the chain had to run longer.



Figure S4. The plots demonstrate the movement of the chains in two dimensional representations of treespace. The space is created using all the chains together, hence, the treespaces of different chains are comparable. The treespace plots for all the chains will looks similar if the chains are sampling similar tree topologies.



Figure S5. The Average Standard Deviation of Split Frequencies (ASDSF) of clades that appear in any of the chains, are calculated from cumulative samples in the chain at various points in the analysis. The solid line shows the ASDSF and the ribbons the 75%, 95% and 100% quantiles of the Standard Deviation of the Split Frequencies (SDSF) across the clades. A lower ASDSF indicates that the chains are more in agreement on split frequencies. The y-axis is plotted on a log10 scale. In general, the SDSF is expected to get lower as the chains progress.

Chart, histogram

Description automatically generated

Figure S6. Split Frequency scatterplot matrix shows the how much agreement is there between regions of treespace the chains have sampled. For each comparison, the cumulative final frequency of each clade in a chain is plotted against the same on another chain. The correlation (Pearson’s R) and ASDSF for each pair of chains is also shown. A satisfactory agreement will have the R-value between 0.9 and 1.0, and the ASDSF value will be lesser than 0.01.   
Chart

Description automatically generated with medium confidence

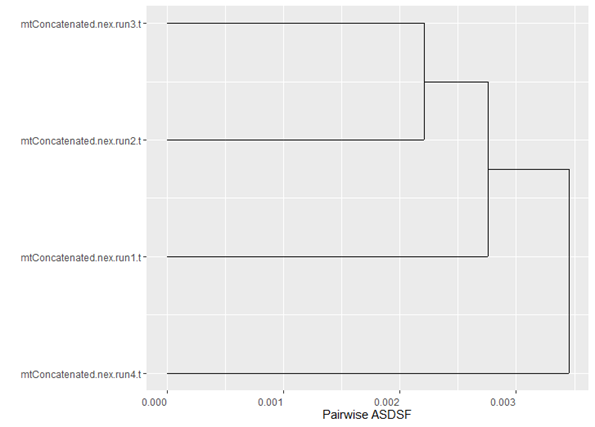
Figure S7. The ASDSF dendogram shows the similarity of split frequencies between chains. The more similar ones are grouped together. This plot helps indicate which chains are sampling similar region of the treespace.   


Figure S8. Bayesian Tree: Branch labels indicate the posterior probabilties (%). In this and subsequent figures, each clade is labelled and designated in a particular colour. These clades are, A. *G. himalayanus* (Black); B. *G. blomhoffi, G. brevicaudus, G. ussuriensis* (Yellow); C. *G. qinlingensis* (Red); D. *G. liupanensis* (Purple); E. G*. halys, G. intermedius, G. changdaoensis, G. cognatus, G. stejnegeri, G. shedaoensis* (Blue); F. *G. angusticeps, G. strauchi, G. monticola, G. rubromaculatus* (Green).

Diagram

Description automatically generated

Table S3. List of Accession Number for the species of Gloydius Hoge & Romano-Hoge, 1981 used in this study. The accession numbers for the sequences produced for this study have been highlighted in yellow. The country and region code are as per the ISO-3166 standard published by the International Organization of Standardisation (<https://www.iso.org/iso-3166-country-codes.html>).

| **Species** | **Country** | **Region** | **Specimen ID** | **cmos** | **PRLR** | **UBN1** | **RAG1** | **NT3** | **Cytb** | **ND4** | **12S** | **16S** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| *Gloydius angusticeps* | CN | SC | JS1708G5C | OP422579 | OP450854 | OP422607 | OP450920 | OP450889 | KY040631 | KY040652 | KY040545 | KY040577 |
| *Gloydius angusticeps* | CN | SC | ROM20473 | KX694784 | -- | -- | -- | KX694986 | -- | AY223620 | KX694594 | -- |
| *Gloydius blomhoffi* | JP | HK | B524 | OP422580 | OP450855 | OP422608 | OP450921 | OP450890 | AY352751 | AY352814 | AY352719 | AY352719 |
| *Gloydius brevicaudus* | CN |  | B525 | -- | OP450856 | -- | OP450922 | OP450891 | AY352752 | AY352815 | AY352781 | AY352720 |
| *Gloydius brevicaudus* | CN | HB | B907 | -- | OP450857 | -- | OP450923 | OP450892 | -- | -- | -- | -- |
| *Gloydius brevicaudus* | CN | LN | DL70 | OP422581 | OP450858 | OP422609 | -- | OP450893 | HQ528467 | HQ528303 | KY040552 | HQ528303 |
| *Gloydius brevicaudus* | CN | ZJ | B906 | OP422582 | OP450859 | OP422610 | -- | -- | -- | -- | OP508264 | EU729426 |
| *Gloydius changdaoensis* | CN | SD | B893 | OP422583 | OP450860 | OP422611 | OP450924 | -- | OP480160 | -- | OP508265 | -- |
| *Gloydius changdaoensis* | CN | SD | B895 | -- | OP450861 | OP422613 | OP450925 | OP450895 | -- | OP407963 | -- | -- |
| *Gloydius changdaoensis* | CN | SD | B894 | -- | -- | OP422612 | -- | OP450894 | -- | -- | -- | -- |
| *Gloydius changdaoensis* | CN | SD | JSSD1510C1 | OP422584 | OP450862 | OP422614 | OP450926 | OP450896 | KX063823 | KX063796 | KY040522 | KY040554 |
| *Gloydius cognatus* | CN |  | L1 | OP422585 | OP450863 | OP422615 | OP450927 | OP450897 | KX063810 | KX063783 | -- | -- |
| *Gloydius cognatus* | CN | NX | JS130947 | -- | -- | -- | -- | -- | KY040622 | KY040643 | KY040532 | KY040564 |
| *Gloydius cognatus* | CN | SC | CIBQY224 | -- | -- | -- | -- | -- | KY040619 | KY040640 | KY040529 | KY040561 |
| *Gloydius halys* | CN |  | DLG11 | OP422586 | OP450864 | OP422616 | OP450928 | OP450898 | KX063805 | KX063778 | -- | -- |
| *Gloydius halys* | CN | HL | JS1407H9 | OP422587 | OP450865 | OP422617 | OP450929 | OP450899 | KY040618 | KY040639 | KY040528 | KY040560 |
| *Gloydius halys* | CN | LN | SYNU1301908 | -- | -- | -- | -- | -- | KX063802 | KX063775 | KY040526 | KY040558 |
| *Gloydius chambensis sp. nov.* | IN | HP | 17.v13 | OP422591 | OP776641 | -- | OP450943 | OP450900 | OP480161 | OP407964 | -- | OP518267 |
| *Gloydius chambensis sp. nov.* | IN | HP | 17.v20 | OP422592 | OP450867 | -- | -- | OP450901 | OP480162 | OP407965 | OP508266 | OP518268 |
| *Gloydius chambensis sp. nov.* | IN | HP | 18.13 | OP422588 | OP450868 | -- | -- | -- | -- | OP407966 | -- | OP518269 |
| *Gloydius chambensis sp. nov.* | IN | HP | 19.38 | OP422593 | OP450871 | OP422618 | OP450930 | OP450902 | OP480163 | OP407969 | OP508267 | OP518271 |
| *Gloydius chambensis sp. nov.* | IN | HP | 19.40 | OP422594 | OP450872 | OP422619 | OP450931 | OP450903 | OP480164 | -- | -- | OP518272 |
| *Gloydius chambensis sp. nov.* | IN | HP | 19.44 | OP422589 | OP450873 | -- | -- | OP450904 | -- | OP407970 | OP508268 | OP518273 |
| *Gloydius chambensis sp. nov.* | IN | HP | 19.50 | OP422595 | OP450874 | OP422620 | OP450932 | OP450905 | OP480165 | OP407971 | OP508269 | -- |
| *Gloydius chambensis sp. nov.* | IN | HP | 18.54 | -- | -- | -- | -- | -- | -- | OP407967 | -- | -- |
| *Gloydius chambensis sp. nov.* | IN | HP | 18.55 | OP422590 | -- | -- | -- | -- | -- | OP407968 | -- | OP518270 |
| *Gloydius himalayanus* | IN | HP | 17.v03 | -- | OP450866 | -- | -- | OP450906 | OP480166 | OP407972 | OP508270 | OP518274 |
| *Gloydius himalayanus* | IN | HP | 18.30 | OP422596 | OP450869 | -- | -- | -- | -- | -- | -- | OP518275 |
| *Gloydius himalayanus* | IN | HP | 18.31 | OP422597 | -- | -- | -- | -- | -- | OP407973 | -- | -- |
| *Gloydius himalayanus* | IN | HP | 19.30 | OP422598 | OP450870 | OP422621 | OP450933 | OP450907 | MZ959173 | MZ959172 | MZ958982 | MZ958980 |
| *Gloydius intermedius* | CN | JL | GP1328 | JQ687521 | OP450875 | -- | -- | -- | JQ687502 | JQ687483 | JX661218 | OP518276 |
| *Gloydius intermedius* | CN | LN | GP191 | JQ687508 | -- | -- | -- | -- | JQ687489 | JQ687470 | JX661219 | -- |
| *Gloydius intermedius* | CN | LN | JS150722 | -- | -- | -- | -- | -- | KY040617 | KY040638 | KY040524 | KY040556 |
| *Gloydius liupanensis* | CN | NX | CHS083 | -- | -- | -- | -- | -- | MK201255 | -- | MK065338 | MK193903 |
| *Gloydius liupanensis* | CN | NX | GP198 | JQ687510 | OP450876 | -- | -- | OP450908 | JQ687491 | JQ687472 | OP508271 | OP518277 |
| *Gloydius liupanensis* | CN | NX | GP206 | JQ687511 | OP450877 | -- | -- | OP450909 | JQ687492 | JQ687473 | OP508272 | -- |
| *Gloydius monticola* | CN | YN | CHS338 | -- | -- | -- | -- | -- | -- | -- | MK065485 | MK194053 |
| *Gloydius monticola* | CN | YN | JS1607DL1 | -- | -- | -- | -- | -- | KY040635 | MG025935 | KY040549 | KY040581 |
| *Gloydius qinlingensis* | CN |  | QL2 | OP422599 | OP450878 | OP422622 | OP450934 | OP450910 | -- | -- | -- | -- |
| *Gloydius qinlingensis* | CN | SN | GP197 | JQ687509 | OP450879 | -- | -- | OP450911 | JQ687490 | JQ687471 | OP508273 | -- |
| *Gloydius qinlingensis* | CN | SN | JS1505QL1 | OP422600 | OP450880 | OP422623 | OP450935 | OP450912 | KY040623 | KY040644 | KY040534 | KY040566 |
| *Gloydius rubromaculatus* | CN | QH | IOZ002317 | OP422601 | OP450881 | OP422624 | OP450936 | OP450913 | KY040632 | KY040654 | KY040654 | KY040578 |
| *Gloydius rubromaculatus* | CN | QH | JS1607Y5 | OP422602 | OP450882 | OP422625 | OP450937 | OP450914 | KY040634 | KY040655 | KY040655 | KY040580 |
| *Gloydius shedaoensis* | CN | LN | B849 | OP422603 | OP450883 | OP422626 | OP450938 | OP450915 | -- | OP407974 | -- | OP518278 |
| *Gloydius shedaoensis* | CN | LN | B856 | OP422604 | OP450884 | -- | OP450939 | OP450916 | OP480167 | OP407975 | OP508274 | OP518279 |
| *Gloydius shedaoensis* | CN | LN | B888 | -- | OP450885 | OP422627 | OP450940 | OP450917 | -- | OP407976 | -- | -- |
| *Gloydius stejnegeri* | CN | BJ | JSSD151054 | OP422605 | OP450886 | OP422628 | OP450941 | OP450918 | KY040625 | KY040646 | KY040539 | KY040571 |
| *Gloydius stejnegeri* | CN | SN | JS1409S3 | -- | -- | -- | -- | -- | KX063817 | KX063790 | KY040536 | -- |
| *Gloydius strauchi* | CN | SC | JSI1410G3 | OP422606 | OP450887 | OP422629 | OP450942 | OP450919 | KY040629 | KY040650 | KY040543 | KY040575 |
| *Gloydius tsushimaensis* | JP |  | TS1 | -- | -- | -- | -- | -- | JN870203 | JN870211 | JN870186 | -- |
| *Gloydius ussuriensis* | CN | HL | U1 | -- | -- | -- | -- | -- | KP262412 | KP262412 | KP262412 | KP262412 |
| *Gloydius ussuriensis* | CN | JL | GP1326 | JQ687520 | OP450888 | -- | -- | -- | JQ687501 | JQ687482 | OP508275 | OP518280 |