A novel SARS-CoV-2 related virus with complex recombination isolated from bats in Yunnan province, China

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Key words: SARS-CoV-2 related virus; Bats; CoVID-19; Recombination; Evolution

28 **Abstract**

29 A novel beta-coronavirus, SARS-CoV-2, emerged in late 2019 and rapidly spread throughout the
30 world, causing the COVID-19 pandemic. However, the origin and direct viral ancestors of
31 SARS-CoV-2 remain elusive. Here, we discovered a new SARS-CoV-2-related virus in Yunnan
32 province, in 2018, provisionally named PrC31, which shares 90.7% and 92.0% nucleotide
33 identities with SARS-CoV-2 and the bat SARSr-CoV ZC45, respectively. Sequence alignment
34 revealed that several genomic regions shared strong identity with SARS-CoV-2, phylogenetic
35 analysis supported that PrC31 shares a common ancestor with SARS-CoV-2. The receptor binding
36 domain of PrC31 showed only 64.2% amino acid identity with SARS-CoV-2. Recombination
37 analysis revealed that PrC31 underwent multiple complex recombination events within the
38 SARS-CoV and SARS-CoV-2 sub-lineages, indicating the evolution of PrC31 from
39 yet-to-be-identified intermediate recombination strains. Combination with previous studies
40 revealed that the beta-CoVs may possess more complicated recombination mechanism. The
41 discovery of PrC31 supports that bats are the natural hosts of SARS-CoV-2.

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50 **Introduction**

51 Coronaviruses (CoVs) are a group of viruses that can infect humans and various
52 mammalian and bird species (1, 2). So far, seven CoV species have been identified in humans. Of
53 these, severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in 2003 and caused
54 multiple epidemics worldwide, and had a fatality rate of ~9.5%(3). Approximately ten years later,
55 another highly pathogenic human CoV, Middle East respiratory syndrome coronavirus (MERS-CoV)
56 emerged and caused numerous outbreaks in the Middle East and South Korea in 2015 (4-6). In
57 December 2019, a novel beta-CoV, now termed severe acute respiratory syndrome coronavirus 2
58 (SARS-CoV-2), was first identified. SARS-CoV-2 caused a pneumonia outbreak in Wuhan, China,
59 and eventually caused a pandemic, with > 116,521,000 reported cases and > 2,589,000 deaths
60 worldwide as of March 9, 2021 (7-10).

61 Both SARS-CoV and MERS-CoV are likely to have originated from bats (5, 10-13). Many
62 SARS-related coronaviruses (SARSr-CoV) have been discovered in bats following CoV outbreaks
63 (11, 14-16), suggesting that bats may be the natural hosts of SARS-CoV. Similarly, several
64 MERS-related coronaviruses have also been isolated from various bat species (5). Notably, palm
65 civets and dromedary camels most likely served as intermediate hosts for SARS-CoV and
66 MERS-CoV, respectively, because these animals carried almost identical viruses to the SARS-CoV
67 and MERS-CoV strains isolated from humans (5). Furthermore, two human coronaviruses,
68 HCoV-NL63 and HCoV-229E, are also considered to have originated in bats, whereas HCoV-OC43
69 and HKU1 were likely to have originated from rodents (5, 17).

70 Since the identification of SARS-CoV-2, CoVs phylogenetically related to SARS-CoV-2
71 (RaTG13, RmYN02, Rc-o319, RshSTT182, RshSTT182200 and RacCS203) have been discovered in
72 bats from China, Japan, and Cambodia (7, 18-23), with most of them discovered by analyzing
73 stored frozen samples (7, 10, 14, 19-22). Of these, RaTG13 and RmYN02, which were identified in
74 Yunnan province, China, shared whole-genome nucleotide sequence identities of 96.2% and 93.3%
75 with SARS-CoV-2, respectively (7, 19). SARS-CoV-2-related CoVs were also identified in pangolins,
76 whose receptor binding domain (RBD) shared up to 97.4% nucleotide identity with that of
77 SARS-CoV-2 (20, 21). This suggests that pangolins are a potential host of SARS-CoV-2, although
78 the role of pangolins in the evolutionary history of SARS-CoV-2 remains elusive. Nevertheless,
79 either the direct progenitor of SARS-CoV-2 is yet to be discovered, or the transmission route of
80 SARS-CoV from bats to humans via an intermediate host must still be determined (24). The
81 discovery of more SARS-CoV-2-related viruses will help to clarify the details regarding the
82 emergence and evolutionary history of SARS-CoV-2.

83

84 **Results**

85 **Identification of a novel SARS-CoV-2-related coronavirus**

86 Based on the molecular identification results, all collected bats belonged to five different
87 species: *Rhinolophus affinis*, *Miniopterus schreibersii*, *Rhinolophus blythi*, *Rhinolophus pusillus*,
88 and *Hipposideros armiger*. By retrospectively analyzing our NGS data, we found a new bat
89 beta-CoV related to SARS-CoV-2 in *Rhinolophus blythi* collected from Yunnan province, China, in
90 2018. The qRT-PCR results revealed that two samples tested positive for SARS-CoV-2 with Ct

values of 32.4 (sample C25) and 35.6 (sample C31). Both bats were identified as *Rhinolophus blythi*. A near complete genome of this virus comprising 29,749 bp was obtained from sample C31 and tentatively named PrC31. The virus genome isolated from the second positive sample had the same sequence as PrC31.

Genetic characteristics and comparison with SARS-CoV-2 and other related viruses

Analysis of the complete PrC31 genome revealed that it shared 90.7% and 92.0% nucleotide identity to SARS-CoV-2 and bat SARSr-CoV ZC45, respectively (Table 1). Although the whole genome of PrC31 was more closely related to ZC45 compared to the other viruses examined, several genes of PrC31 showed highly similar nucleotide identities (> 96%) with SARS-CoV-2, including E, ORF7a, ORF7b, ORF8, N and ORF10 (Table 1). Notably, ORF8 and ORF1a (the region spanning nucleotides 1–12719) of PrC31 were genetically closer to SARS-CoV-2 than any other viruses identified to date, exhibiting 98.1% and 96.6% nucleotide identities, respectively. However, in other regions, PrC31 was more similar to SARS-CoV or SARSr-CoV ZC45.

The RBD of PrC31 was evolutionarily distant from SARS-CoV-2, sharing only 64.2% amino acid identity, whereas it was almost identical to that of ZC45, with only one amino acid difference. Similar to most bat SARSr-CoVs, one long (14 aa) deletion and one short (5 aa) deletion were present in PrC31, which were absent from SARS-CoV, SARS-CoV-2, pangolin-CoV and RaTG13. We predicted the three-dimensional structure of the RBD of PrC31, ZC45 and SARS-CoV-2 using homology modeling. Similar to RmYN02, the two loops close to the receptor binding site of the

112 PrC31 RBD were shorter than those of SARS-CoV-2, due to two deletions; this region may
113 influence the binding capacity of the PrC31 RBD with the angiotensin converting enzyme 2 (ACE2)
114 receptor (Fig.1A-1D). Moreover, of the six amino acid residues that are essential for the binding
115 of the SARS-CoV-2 spike protein to ACE2 (L455, F486, Q493, S494, N501, and Y505), PrC31 and
116 RmYN02 possessed only one(Y505) (Fig.1E)

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118 Phylogenetic analysis of PrC31 and representative sarbecoviruses

119 Phylogenetic analysis of the complete PrC31 genome revealed that it belonged to a
120 separate clade to SARS-CoV-2, while most other SARS-CoV-2-related viruses were grouped
121 together (Fig.2). However, the PrC31 RNA-dependent RNA polymerase was phylogenetically
122 grouped within the SARS-CoV lineage and clustered with bat SARS-rCoV. The spike protein of
123 PrC31 fell within the SARS-CoV-2 sub-lineage and clustered with ZC45 and CXZ21, while being
124 distant from SARS-CoV-2. The topological differences between various regions of PrC31 strongly
125 suggest the occurrence of recombination events throughout its evolution.

126

127 Multiple and complex recombination events in the evolution of PrC31

128 The full-length genome sequences of PrC31 and closely related beta-CoVs were aligned to
129 search for possible recombination events. Strikingly, both the similarity and bootstrap plots
130 revealed multiple and complex long-segment recombination events in PrC31, which likely arose
131 from multiple beta-CoVs from within the SARS-CoV and SARS-CoV-2 sub-lineage. As shown in
132 Figure 3, three recombination breakpoints were detected. For the region spanning nucleotides

133 1–12,719 and 27,143 to the 3' terminus of the genome, PrC31 was most closely related to
 134 SARS-CoV-2 and RmYN02. In these regions, PrC31 was phylogenetically grouped with RmYN02
 135 and in a sister clade to SARS-CoV-2 (Figure 4a and 4d). For the 12,720–20,244 nucleotide region,
 136 which included ORF1ab, PrC31 was grouped with SARS-CoV and bat SARSr-CoVs (Figure 4b).
 137 Moreover, PrC31 presented the highest similarity to ZC45 in the 20,245–27,142 genomic
 138 fragment, which included part of ORF1ab, S, ORF3, E, and part of the M gene, and fell within the
 139 SARS-CoV-2 sub-lineage (Figure 4c)

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141 Discussion

142 The recently-emerged SARS-CoV-2 virus triggered the ongoing COVID-19 pandemic, which
 143 has high morbidity and fatality rates, and poses a great threat to global public health.
 144 Identifying the origin and host range of SARS-CoV-2 will aid in its prevention and control, and will
 145 facilitate preparation for future CoV pandemics. Although several SARS-CoV-2-related viruses
 146 were detected in bats and pangolins, none of them appear to be the immediate ancestor of
 147 SARS-CoV-2; the exact origin of SARS-CoV-2 is still unclear (12, 25). In this study, we discovered
 148 PrC31, a sarbecovirus isolated from bat intestinal tissues collected in 2018. PrC31
 149 phylogenetically falls into the SARS-CoV-2 clade and has undergone multiple and complex
 150 recombination events.

151

152 Animals that continuously harbor viruses closely related to SARS-CoV-2 for extended time
 153 periods can become natural SARS-CoV-2 hosts (2). To date, several bat viruses have been

154 identified that have strong sequence similarities to SARS-CoV-2, sharing more than 90% sequence
155 identity. Especially, RaTG13 possesses 96.2% identity with SARS-CoV-2 (7, 18, 19, 21, 23). The
156 PrC31 virus identified in this study showed 90.7% genome identity with SARS-CoV-2; notably, the
157 E, ORF7, ORF8, N and ORF10 genes shared more than 96% identity with SARS-CoV-2. Both the
158 genetic similarity and diversity of SARS-CoV-2-related viruses support the claim that bats were
159 the natural hosts of SARS-CoV-2 (10, 19).

160

161 Recombination events between various SARSr-CoVs have occurred frequently in bats (5, 16).
162 SARS-CoV-2 may also be a recombined virus, potentially with the backbone of RaTG13 and a RBD
163 region acquired from pangolin-like SARSr-CoVs (12, 21). In this study, we found that PrC31
164 phylogenetic clustered with SARS-CoV-2 and its related viruses. The results from our phylogenetic
165 analyses suggested that recombination had occurred in PrC31. The similarity plot indicated that
166 the PrC31 was subjected to multiple and complex recombination events involving more than two
167 sarbecoviruses in the SARS-CoV and SARS-CoV-2 sub-lineages. The three breakpoints of PrC31
168 separate the genome into four regions. Region 1 (within ORF1a) and region 4 of PrC31 were
169 closely related to SARS-CoV-2, RaTG13 and RmYN02. Region 2 of PrC31 was more similar to
170 members of the SARS-CoV sub-lineage, including SARS-CoV and SARSr-CoV Rs4237 strain; region
171 3 was more closely related to ZC45 within SARS-CoV-2 sub-lineage. The multiple recombination
172 events of PrC31 hint toward the existence of intermediate recombination strains within the
173 SARS-CoV and SARS-CoV-2 sub-lineages that are yet to be identified. Our work suggests that the
174 backbone of PrC31 may have evolved from a recent common ancestor of RaTG13, RmYN02 and

175 SARS-CoV-2, and that it acquired regions 2 and 3 from precursor viruses of SARS-CoV and
176 SARSr-CoV ZC45, respectively.

177 At present, the precise patterns and mechanisms driving recombination in sarbecoviruses are
178 largely unknown. A recent report identified 16 recombination breakpoints in 69 sarbecoviruses
179 (26), although in the majority of strains, the recombination sites were located within the S gene
180 and upstream of ORF8 (5, 9, 16). The three recombination breakpoints of PrC31 were located in
181 ORF1a, ORF1b and M genes with long fragment recombination, suggestive of a complicated
182 recombination pattern in sarbecoviruses. Similar to PrC31, SARS-CoV-2 may have evolved via
183 complex recombination between various related coronaviruses or their progenitors (10). In fact,
184 the direct progenitor of SARS-CoV may have evolved by recombination with progenitors of
185 SARSr-CoV (Hu et al. 2017). Together, these findings suggest that recombination and its role in
186 the evolution history of sarbecoviruses may be more complicated and significant than initially
187 expected.

188 Pangolins may also harbor ancestral beta-CoVs related to SARS-CoV-2 (2, 20, 21); the
189 receptor-binding motif of pangolin beta-CoVs share an almost identical amino acid sequence with
190 SARS-CoV-2 (20, 21), suggesting that SARS-CoV-2 may have acquired its RBD region from a
191 pangolin CoV via recombination(27). However, unlike bats, pangolins infected with beta-CoVs
192 present overt symptoms and eventually die, rendering them unlikely to be natural hosts.
193 Intermediate hosts generally serve as zoonotic sources for human infection, acting as vectors for
194 viral replication and transmission to humans (2). Current evidence suggests that pangolins were
195 not the direct intermediate hosts of SARS-CoV-2. However, pangolins certainly played an

196 important role in the evolutionary history of SARS-CoV-2 related viruses, eventually leading to
197 the transmission of SARS-CoV-2 to humans. It cannot be excluded that a novel recombination
198 event involving SARS-CoV-2 or SARS-CoV-2 related viruses and SARS-CoV or SARSr-CoV will lead
199 to the virus presumed as “SARS-CoV-3”, which may be transmitted to human populations in the
200 future.

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202 The discovery of PrC31 provides more evidence for the bat origin of SARS-CoV-2 (10, 28).
203 Identifying more SARS-CoV-2 related viruses in nature will provide deeper insight into the origins
204 of SARS-CoV-2. It will be necessary to expand the sampling areas and animal species examined to
205 find more close relatives of SARS-CoV-2. There may be an unknown intermediate host of
206 SARS-CoV-2 that played a similar role to that of civets and camels in the SARS-CoV and MERS-CoV
207 epidemics, respectively. Furthermore, PrC31 was firstly tested for positive using SARS-CoV-2 qPCR
208 kit, which targets the ORF1ab and N genes of SARS-CoV-2. This emphasizes the need to gather
209 sequence information for positive samples during environmental surveillance of SARS-CoV-2, as
210 samples may be contaminated with a closely related beta-CoVs from wild animals such as bats.

211

212 **Materials and methods**

213 We retrospectively analyzed bat next generation sequencing (NGS) data that we
214 performed in 2019, and found SARS-CoV-2-related reads present in one pool of intestinal tissues.
215 The details of sampling and high-throughput sequencing are given below.

216

217 **Sample collection and pretreatment**

218 In 2018, 36 bats were captured in Yunnan province, China. The bats were dissected following
219 anesthetization. Liver, lung, spleen and intestinal tissue specimens were collected and
220 transported to the Chinese center for disease control, where they were stored at -80°C until
221 further analysis. The bat species were identified by polymerase chain reaction (PCR) to amplify
222 the cytochrome B gene, as previously described (29). Intestinal tissues collected from 36 bats
223 were homogenized in minimum essential medium and the suspensions were centrifuged at 8,000
224 rpm. The supernatants were merged into two pools according to bat species, then digested using
225 DNase I for RNA Extraction. All procedures were performed in a biosafety cabinet in a biosafety
226 level 2 facility. This study was approved by the ethics committee of the CCDC, and was performed
227 according to Chinese ethics, laws and regulations.

228

229 **RNA extraction and next-generation sequencing (NGS)**

230 Nucleic acids were extracted using a QIAamp MinElute Virus Spin Kit (QIAGEN) and used to
231 construct the sequencing libraries. The library preparation and sequencing steps were performed
232 by Novogene Bioinformatics Technology (Beijing, China). In brief, the ribosomal RNA was
233 removed using the Ribo-Zero-Gold (Human–Mouse–Rat) Kit (Illumina, USA) and the
234 Ribo-Zero-Gold (Epidemiology) Kit (Illumina). The libraries were constructed using a Nextera XT
235 kit (Illumina), and sequencing was performed on the Illumina NovaSeq 6000 platform according
236 to the procedure for transcriptome sequencing.

237

238 **Bioinformatic analyses**

239 Bioinformatics analysis of the sequencing data was conducted using an in-lab bioinformatics
240 analysis platform. Prinseq-lite software (version 0.20.4) was used to remove lower quality reads,
241 and Bowtie2 was used to align and map the filtered reads to the host reference genome. Mira
242 (version 4.0.2) was used for *de novo* assembly of the clean reads. Both BLASTn and BLASTx of the
243 BLAST+ package (version 2.2.30) were used to search against local viral nucleotide and protein
244 databases. The E-value cut-off was set to 1×10^{-5} to maintain high sensitivity and a low
245 false-positive rate when performing BLAST searches.

246

247 **Sequencing of full-length genomes and quantitative real-time PCR (qRT-PCR)**

248 We obtained reads that showed 96–98% nucleotide identity to SARS-CoV-2 from the PrC31
249 genome library. To confirm the sequences obtained from NGS and to fill the gaps, we designed 32
250 primer pairs according to the consensus sequences from the NGS and the conserved regions of
251 SARS-CoV-2, RaTG13 and RmYN02, to amplify the whole PrC31 genome with at least 100 bp
252 overlap between adjacent PCR fragments (Table S1). The PCR products were subjected to Sanger
253 sequencing with pair-end sequencing. The 25 bp at the 5' and 3' termini were omitted, and the
254 remaining sequences were assembled using Geneious Prime. Positive samples were quantified
255 using TaqMan-based qPCR kit targeting the ORF1ab and N genes (BioGerm, China).

256

257 **Phylogenetic and recombination analyses**

258 The complete genome sequences of reference viruses were downloaded from GenBank

(<https://www.ncbi.nlm.nih.gov/>) and GISAID (<https://www.gisaid.org/>). The complete genome of PrC31 was aligned with representative SARS-CoV, SARS-CoV-2 and SARSr-CoV using Mafft (v7.475). Phylogenetic analyses were performed with RaxML software (v8.2.11) using the general time reversible nucleotide substitution model, GAMMA distribution of rates among sites, and 1000 bootstrap replicates. Potential recombination events were screened using RDP4 software and further analyzed by similarity plot using Simplot (v3.5.1) with potential major and minor parents.

266

267 **Structural modeling**

268 The three-dimensional structures of PrC31, ZC45 and SARS-CoV-2 RBDs were modeled with the Swiss-Model program using the SARS-CoV-2 RBD structure (PDB: 7a91.1) as the template.

270

271 **Data availability**

272 The sequences of PrC31 generated in this study were deposited in the GISAID and GenBank databases with the accession numbers EPI_ISL_1098866 and MW703458, respectively.

274 **Acknowledgements**

275 This study was supported by National Science and Technology Major Project of China
276 (No.2018ZX10305409-004-002)

277 **Author contributions**

278 L-L L, Acquisition of data, Analysis and interpretation of data, Conception and design, Drafting or
279 revising the article; M-XH, Acquisition of data; J-S L, Conception and design experiment; J-L W,
280 Sample collection, Acquisition of data. W-F S, Analysis and interpretation of data, Conception and
281 design, Drafting or revising the article. Z-J D, Conception and design, Analysis and interpretation

282 of data, Drafting or revising the article.

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285 Table 1: Sequence identities comparing PrC31 with SARS-CoV-2 and other representative

286 beta-CoVs

		Complete	Genes											
Strain		Genome	1ab	S	RBD	3a	E	M	6	7a	7b	8	N	10
nt	Wuhan-Hu-1	90.7	92.6	74.9	61.5	89.2	99.1	93.4	94.4	96	97.8	98.1	97	99.1
(%)	RaTG13	90.4	92	75.6	61.6	88.7	98.7	93.3	95.9	93.8	98.5	97.8	96.6	98.3
	ZC45	92	91.2	94.8	95.3	95.6	98.7	96	91.8	87.9	95.6	89.4	91.3	98.3
	RmYN02	90.4	93.2	74.3	81.1	88.5	97.8	91	95.4	95.4	93.3	48.8	98.1	98.3
	Pangolin/GXP5L	83.3	83.5	75.2	61.9	85.1	96.5	90.9	89.3	85.5	84.4	81	91.2	94.9
	Pangolin/GD	87.9	90.3	79.2	61.2	90.6	98.3	93.1	91.3	91.7	94.1	93.2	96.2	98.3
	Rc-o319	79.3	80.3	70.7	63.8	79.3	96.5	84.8	85.2	77.2	79.3	46.3	87.7	95.7
	Rs4237	82.3	83.2	74.5	82.1	75.6	92.2	82.7	76.1	82.8	80.7	65.8	87.9	92.3
	Tor2	81.4	83	71.6	63.7	74.5	92.6	83.4	74.6	80.6	80.7	44.6	88.1	93.2
	RShSTT182	88.6	90.3	72.2	62.9	87.6	98.3	90.0	87.6	94.4	99.3	95.1	94.3	98.3
	RacCS203	88.8	90.3	74.4	80.1	87.8	98.2	92.5	91.4	90.8	93.2	92.9	93.7	99.1

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294 **Figure legends**

295 **Figure 1: Homology modeling structures and Characterization of Receptor binding domain (RBD)**

296 **of PrC31 and Representative Beta-CoVs. (A-D) Homology modeling structures of PrC31 and**

297 **Representative Beta-CoVs. The three-dimensional structures of PrC31, ZC45 and SARS-CoV-2**

298 **RBDs were modeled using the Swiss-Model program, using the SARS-CoV-2 RBD structure (PDB:**

299 **7a91.1) as a template. The two deletion loops in PrC31 and ZC45 are marked with a circle. (E)**

300 **Characterization of the RBDs of PrC31 and representative beta-CoVs. The six critical amino acid**

301 **residues for ACE2 interaction were marked using red star.**

302

303 **Fig. 2 Phylogenetic trees of SARS-CoV-2 and representative sarbecoviruses. (A)Complete genome;**

304 **(B)RdRp gene (C)S gene (D)RBD region. SARS-CoV lineage and SARS-CoV-2-related lineages are**

305 **shown in orange and purple shadow, respectively. Viruses that originated in bats are labeled in**

306 **blue, human viruses are labeled in red and pangolin viruses are labeled in green. The PrC31**

307 **identified in this study is highlighted in yellow shadow. Phylogenetic analyses were performed**

308 **with RaxML software (v8.2.11) using the GTR nucleotide substitution model, GAMMA distribution**

309 **of rates among sites, and 1000 bootstrap replicates**

310

311 **Fig. 3 Recombination analysis. A. Genome organization of PrC31. (B) Similarity plot and (C)**

312 **Bootstrap plot of full-length genome of human SARS-CoV-2, pangolin- and bat beta-CoVs using**

313 PrC31 as the query. Slide window was set to 1000 bp with 100 bp steps.

314

315 Fig. 4 Phylogenetic trees of various regions of the PrC31 genome. SARS-CoV and
316 SARS-CoV-2-related lineages are shown as orange and purple shadow, respectively. The PrC31
317 virus identified in this study is indicated with yellow shadow. Viral taxonomy is labeled in color
318 that originated in bats are labeled in blue, humans in red, and pangolins in green. Phylogenetic
319 analyses were performed with RaxML software (v8.2.11) using the GTR nucleotide substitution
320 model, GAMMA distribution of rates among sites, and 1000 bootstrap replicates

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395

Fig.1

Fig.2

A: Complete genome

B: RdRp gene

C: S gene

D: RBD

Fig.3

Window: 1000 bp, Step: 100 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2.0, Neighbor-Joining

Fig.4

A: Region 1

B: Region 2

C: Region 3

D: Region 4