

1 **MANUSCRIPT TITLE**

2 Telomeric repeat evolution in the phylum Nematoda revealed by high-quality genome
3 assemblies and subtelomere structures

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16 A running title: Telomeric repeat evolution in Nematoda

17

18 ABSTRACT

19 Telomeres are composed of tandem arrays of telomeric-repeat motifs (TRMs) and telomere-
20 binding proteins (TBPs), which are responsible for ensuring end-protection and end-
21 replication of chromosomes. TRMs are highly conserved due to the sequence specificity of
22 TBPs, although significant alterations in TRM have been observed in several taxa, except
23 Nematoda. We used public whole-genome sequencing datasets to analyze putative TRMs of
24 100 nematode species and determined that three distinct branches included specific novel
25 TRMs, suggesting that evolutionary alterations in TRMs occurred in Nematoda. We focused
26 on one of the three branches, the Panagrolaimidae family, and performed a *de novo*
27 assembly of four high-quality draft genomes of the canonical (TTAGGC) and novel TRM
28 (TTAGAC) isolates; the latter genomes revealed densely clustered arrays of the novel TRM.
29 We then comprehensively analyzed the subtelomeric regions of the genomes to infer how
30 the novel TRM evolved. We identified DNA damage–repair signatures in subtelomeric
31 sequences that were representative of consequences of telomere maintenance mechanisms
32 by alternative lengthening of telomeres. We propose a hypothetical scenario in which
33 TTAGAC-containing units are clustered in subtelomeric regions and pre-existing TBPs
34 capable of binding both canonical and novel TRMs aided the evolution of the novel TRM in
35 the Panagrolaimidae family.

36

37 INTRODUCTION

38 Since linear chromosomes originated from a circular structure, a specific DNA–protein
39 complex has evolved to protect the ends of the chromosomes, i.e. the telomere. Telomeres
40 are typically composed of tandem arrays of telomeric-repeat motifs (TRMs) and telomere-
41 binding proteins (TBPs) that can bind to the tandem arrays (Zhong et al. 1992). TBP binding
42 interferes with the accession of other proteins. Hence, proteins that respond to DNA damage
43 signals cannot bind to the exposed ends of linear chromosomes and the exposed ends are
44 not recognized as DNA damage sites (Van Steensel et al. 1998; De Lange 2009; Lazzerini-
45 Denchi and Sfeir 2016). This enables the telomere to maintain chromosome integrity.

46 TRM sequences are typically well conserved because TBPs attach to arrays of TRMs in a
47 sequence-specific manner. However, dozens of variant TRMs have evolved in many taxa,
48 including ≥ 150 motifs in fungi (Červenák et al. 2021) and ≥ 7 motifs in plants (Peska and
49 Garcia 2020). Furthermore, except Hymenoptera, many arthropod species have unique
50 telomere structures. For example, most Insecta species have telomere structures composed
51 of telomere-specific retrotransposons interposed between canonical TRMs, whereas Diptera
52 species have telomeres that are exclusively composed of retrotransposons and lack TRMs
53 (Abad et al. 2004; Garavís et al. 2013; Zhou et al. 2022). It is yet to be elucidated how TRMs
54 have been altered while interacting with TBPs. These diverse TRMs and telomere structures
55 developed from the co-evolution of a TRM and several TBPs (Shakirov et al. 2009; Steinberg-
56 Neifach and Lue 2015; Sepsiova et al. 2016; Červenák et al. 2019); however, they may have
57 also originated from TRM-specific changes regardless of TBP changes if TBPs were capable
58 of binding to several types of TRMs (Rotková et al. 2004; Fajkus et al. 2005; Kramara et al.
59 2010; Visacka et al. 2012; Červenák et al. 2019; Tomáška et al. 2019; Červenák et al. 2021).

60 We focused our analysis on subtelomeric regions and the DNA damage–repair signatures
61 generated during telomere evolution, especially telomere and subtelomere reconstruction by
62 alternative lengthening of telomeres (ALT) mechanisms (Heaphy et al. 2011; Sobinoff and
63 Pickett 2017). ALT, which refers to alternative mechanisms that substitute telomerase
64 function for telomere maintenance at the chromosome ends (Bryan et al. 1995), is crucial in
65 telomerase mutant eukaryotic cells such as those in yeast, *Caenorhabditis elegans*, mouse,
66 and human, and can thus be used to model and interpret the evolution of TRM changes
67 (Lundblad and Blackburn 1993; Bryan et al. 1995; Seo et al. 2015; Kim et al. 2020; Kim et al.
68 2021a; Kim et al. 2021b). In these ALT models, homology-directed repair (HDR) mechanisms
69 can add new telomeric components. HDRs exploit the homology between shortened
70 telomeric sequences and tandem arrays of TRMs in other genomic loci to replicate the
71 tandem array and adjacent sequences to the shorter end (Lydeard et al. 2007; Roumelioti et
72 al. 2016; Kramara et al. 2018; Kim et al. 2020). Furthermore, a unique sequence inserted
73 between TRMs, known as the template for ALT, may be used to repair shorter telomeric
74 repeats even in wild-type *C. elegans*, resulting in the formation of a new subtelomeric
75 structure (Kim et al. 2019a). These findings suggest that ALT mechanisms play an essential
76 role in the evolution of telomeres and the evolutionary route can be reconstructed by
77 studying ALT activity traces left in subtelomeric sequences.

78 The present study explored TRM evolution in Nematoda using public whole-genome
79 sequencing (WGS) data obtained from 100 nematode species and our high-quality genome
80 assemblies. We evaluated the subtelomeric regions of TTAGGC-telomere isolates of
81 Panagrolaimidae to infer possible evolutionary paths toward TTAGAC telomeres.

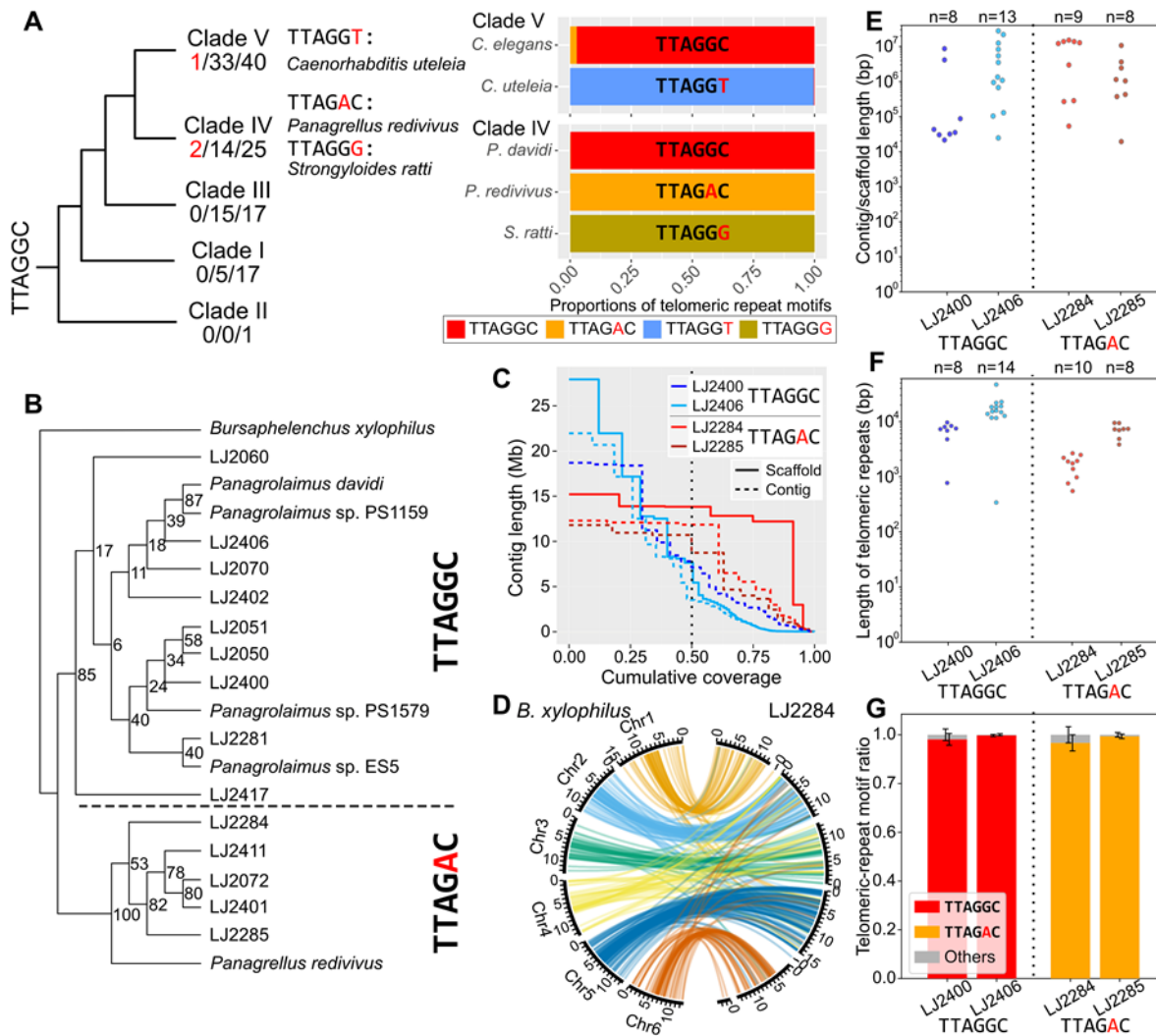
82

83 **RESULTS**

84 **Identification of three novel TRMs in Nematoda**

85 We examined variations in TRMs using public short-read WGS data from Nematoda species
86 that have matched public genome assemblies from all five major clades. A total of 100
87 species met the criteria, including at least 17 species from Clades I, III, IV, and V but only one
88 from Clade II (Fig. 1A). The species and accession numbers used in this analysis are listed in
89 Supplemental Table S1 and S2.

90



91

92

93 **Figure 1.** Putative novel TRMs in the phylum Nematoda and validation of the emergence of

94 TTAGAC in the family Panagrolaimidae. (A) The cladogram was adapted and modified from

95 Smythe et al. 2019 (Smythe et al. 2019) with permission from BMC Ecology and Evolution

96 2019, CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>). The three numbers

97 separated by '/' under each clade indicates the number of species with novel TRMs/the

98 number of species whose TRM was identified in our analysis/total number of species we

99 analyzed, respectively. Changes from TTAGGC, the canonical Nematoda TRM, to the novel

100 TRMs are highlighted in red. Below each novel TRM is a species name with the
101 corresponding TRM. Each bar indicates the proportion of the canonical Nematoda TRM
102 (TTAGGC) and novel TRMs in each species. The upper bar of each pair represents a control
103 species that harbors the canonical TRM. The TRMs are represented as follows: red, TTAGGC;
104 orange, TTAGAC; blue, TTAGGT; khaki, TTAGGG. (B) Phylogenetic relationships of 19
105 Panagrolaimidae species/isolates based on their 18S rDNA sequences and their putative
106 TRMs. *Bursaphelenchus xylophilus* was used as an outgroup species. The number on each
107 node represents the bootstrap support value. (C) Contig/scaffold length distributions of the
108 genome assemblies. The vertical dotted line indicates N50 contig/scaffold size. (D) Synteny
109 plot of LJ2284 compared to *B. xylophilus*. Each colored line represents a BUSCO gene shared
110 among the genome assemblies of our four isolates and *B. xylophilus*. Orange lines indicate
111 that corresponding BUSCO genes are in Chromosome 1 in *B. xylophilus*. Sky blue, bluish
112 green, yellow, blue, and vermillion represent Chromosomes 2, 3, 4, 5, and 6, respectively. We
113 used Wong's color palette designed for color-blind individuals (Wong 2011). (E–G) The
114 vertical dotted line separates the TTAGGC-telomere isolates (left) and the TTAGAC-telomere
115 isolates (right). (E) Length distributions of the contigs/scaffolds containing highly clustered
116 telomeric repeats at the end. Each dot represents each contig/scaffold, and the total number
117 of contigs/scaffolds of each isolate (n) is indicated at the top of the graph. (F) Estimated
118 length distributions of clustered telomeric repeats at the end of the contigs/scaffolds. Each
119 dot indicates a telomeric-repeat cluster for each contig/scaffold, and the total number of
120 telomeric-repeat clusters for each isolate (n) is indicated at the top of the graph. (G) The

121 proportion of TRM types in clustered telomeric repeats. Error bars represent the standard
122 deviation for all clustered telomeric repeats at the end of contigs/scaffolds in each isolate.

123

124 The canonical TRM of Nematoda is known to be a 6-bp sequence (TTAGGC) that covers
125 a >1-kb length in *C. elegans* telomeres (Kim et al. 2019a; Yoshimura et al. 2019). We
126 examined the WGS data from 100 species to identify novel TRMs with a concatemer of 5–7
127 bp, a high copy number, and a pattern similar to TTAGGC. We determined the TRM in 67
128 species using the following criterion: TRM repeats should be long concatemers for TBP
129 binding, so TRM-like *k*-mer counts should be high enough (≥ 100 in 5 million short reads in
130 our analysis) (Fig. 1A and Supplemental Table S1). However, 26 of the remaining 33 species
131 exhibited low *k*-mer counts of TRM-like motifs, so we could not determine their TRMs
132 (Supplemental Table S2). The remaining seven species had high *k*-mer counts, but we
133 excluded them from the TRM-determined group because of the following reasons: we
134 detected potential sample or host contamination in *Enoplus brevis*, two *Strongyloides*, and
135 three *Trichinella* species, and *Diploscapter pachys* had only short concatemers (≤ 10 copies)
136 in the middle of its reads (Supplemental Table S2; see Supplemental Note for more
137 information).

138 TTAGGC, the canonical Nematoda TRM, was identified in 64 of the 67 TRM-determined
139 species (Fig. 1A and Supplemental Table S1). We determined that the other three nematode
140 species had few TTAGGC copies but a high copy number of one of three putative TRMs:
141 TTAGGT, TTAGAC, and TTAGGG in *Caenorhabditis uteleia*, *Panagrellus redivivus*, and

142 *Strongyloides ratti*, respectively (Fig. 1A, Supplemental Fig. S1 and Supplemental Tables S1
143 and S3; see Supplemental Note and Supplemental Table S4 for further validation of the
144 TRMs). The identified TRMs were new to Nematoda and had not been reported previously.
145 We assumed that these three motifs evolved independently because they occurred in
146 phylogenetically distant branches.

147 **The emergence of a new TRM, TTAGAC, in the family Panagrolaimidae**

148 We focused on the TTAGAC sequence of the family Panagrolaimidae to confirm the
149 noncanonical TRM sequence identified by short-read WGS data because many different
150 isolates of this family were widely isolated from regions across the Republic of Korea. Some
151 were easily cultured under laboratory conditions. We used 19 short-read WGS datasets of
152 the family Panagrolaimidae to determine whether the unique motif TTAGAC emerged once
153 or multiple times (Supplemental Table S5). WGS datasets of five species were available from
154 publicly accessible databases, whereas the remainder were derived from sequencing data of
155 the isolates collected from the Republic of Korea, which were probably distinct species as
156 they had distinguishable ribosomal DNA (rDNA) sequences (Supplemental Table S6). *B.*
157 *xylophilus* was also used as the outgroup species.

158 We determined that *Panagrellus redivivus* and five out of fourteen collected isolates had the
159 novel TRM, TTAGAC, whereas all four *Panagrolaimus* species and the remaining nine isolates
160 possessed the canonical TRM, TTAGGC. Moreover, the five collected TTAGAC-TRM isolates
161 clustered with *Panagrellus redivivus*; however, the nine TTAGGC-TRM isolates were grouped
162 with the *Panagrolaimus* species (Fig. 1B and Supplemental Table S5). Furthermore, the

163 TTAGGC concatemer was not observed in any of the 6 TTAGAC-TRM species/isolates, nor
164 was the TTAGAC concatemer found in any of the 13 TTAGGC-TRM species/isolates. These
165 results suggest that TTAGAC sequences in Panagrolaimidae may have arisen from a single
166 ancestor that had TTAGAC as its TRM.

167 **High-quality genome assemblies confirmed that TTAGAC repeats were clustered at the**
168 **ends of contigs or pseudo-chromosome molecules in TTAGAC-TRM isolates.**

169 We constructed *de novo* genome assemblies of Panagrolaimidae isolates using PacBio high-
170 fidelity (HiFi) long-read sequencing technology and Arima Hi-C technology to assess
171 whether the newly identified TRM presented clusters at the ends of the chromosomes. Two
172 TTAGAC-TRM isolates, LJ2284 and LJ2285, and two TTAGGC-TRM isolates, LJ2400 and
173 LJ2406, were selected for the study. We produced 3.1 Gb (43×), 2.9 Gb (55×), 17.4 Gb (26×),
174 and 36.1 Gb (93×) HiFi reads for LJ2284, LJ2285, LJ2400, and LJ2406, respectively. The HiFi
175 reads were then assembled into high-quality draft genomes in which the length of the
176 longest contig and contig N50 varied from 11.80 Mb to 21.96 Mb and 3.28 Mb to 11.84 Mb,
177 respectively. We also generated Hi-C data for LJ2284 and LJ2406 and scaffolded their contigs
178 into larger chunks (Supplemental Fig. S2 and Supplemental Table S7). The longest scaffold
179 lengths increased to 15.2 Mb and 27.9 Mb, and their scaffold N50 increased to 13.8 Mb and
180 7.6 Mb for LJ2284 and LJ2406, respectively (Fig. 1C and Supplemental Table S8). Additionally,
181 91.4% of the LJ2284 contigs were connected as five scaffolds (Supplemental Fig. S2).

182 All of our genome assemblies had ~75% BUSCO completeness, which was comparable to
183 that of the nearly complete genome assembly of *B. xylophilus* (71%) (Supplemental Fig. S3)

184 (Dayi et al. 2020). In addition, we analyzed the synteny relationships between *B. xylophilus*
185 and our genome assemblies using their common single-copy orthologs. Pseudo-
186 chromosome-level scaffolds of LJ2284 and contigs of LJ2285 exhibited marked collinearity
187 for all the chromosomes, except Chromosome 4 of *B. xylophilus* (Fig. 1D, Supplemental Fig.
188 S4 and Supplemental Note). Chromosomes 5 and 6 of *B. xylophilus* also showed collinearity
189 with clusters of LJ2400 contigs and LJ2406 scaffolds, but the collinearity of other
190 chromosomes was much weaker (Supplemental Fig. S4 and Supplemental Note). Based on
191 these findings, our Panagrolaimidae genome assemblies were highly contiguous.

192 The constructed high-quality genome assemblies revealed that the telomeric repeats of both
193 LJ2284 and LJ2285 changed from TTAGGC to TTAGAC. First, we confirmed whether the
194 telomeric repeats had been adequately assembled. Our analysis revealed that each genome
195 assembly had 8–13 contigs/scaffolds ending with telomeric repeats (Fig. 1E). Among these
196 contigs/scaffolds, one each of LJ2284 and LJ2406 was assembled at the telomere-to-
197 telomere level (Supplemental Tables S7 and S8). The telomeric repeats at the ends of the
198 contigs/scaffolds in each genome assembly exhibited various ranges of length in the
199 Panagrolaimidae isolates (Fig. 1F and Supplemental Table S9). Their lengths in LJ2284 ranged
200 from 0.5 to 2.7 kb, but they were much longer in LJ2285 and LJ2400, ranging from 3.8 to 9.7
201 kb (except for one in LJ2400). LJ2406 exhibited telomeric-repeat clusters longer than 10 kb
202 (except for one cluster). We identified that 84.9%–99.4% of raw HiFi reads were longer than
203 10 kb in all four isolates and that LJ2406, which has much longer telomeres than the other
204 three isolates, had 328 reads consisting only of 10–26-kb telomeric repeats (Supplemental
205 Fig. S5). Therefore, the TRM cluster lengths at each contig/scaffold end would be highly

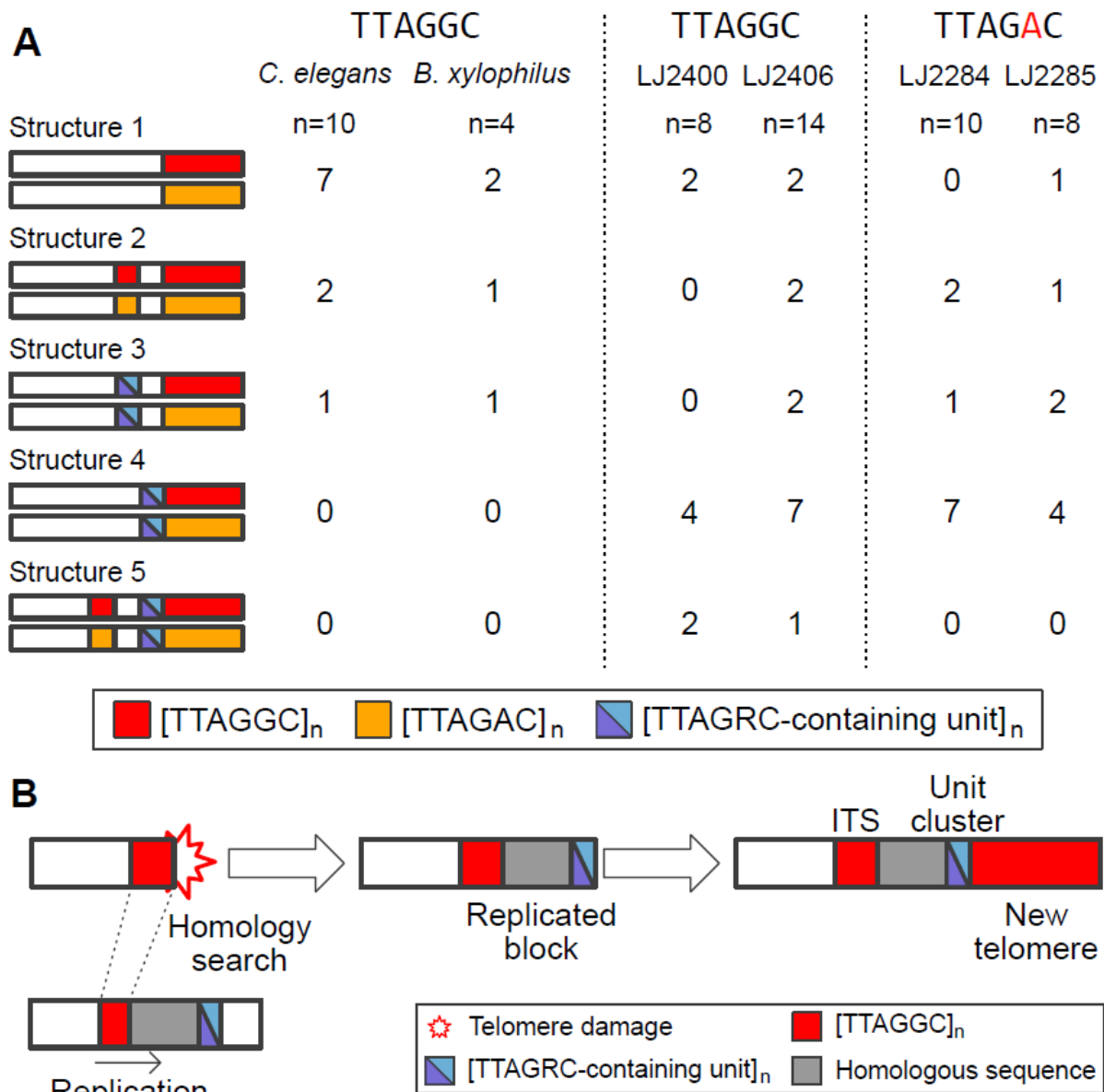
206 correlated with their actual lengths (see Supplemental Note for detailed description). The
207 lengths of the clustered TRMs were comparable to those of the two *C. elegans* assemblies
208 constructed using long-read sequencing technologies (2.3–5.7 kb) (Kim et al. 2019a;
209 Yoshimura et al. 2019). Furthermore, the telomeric repeats consisted mainly of TTAGAC in
210 LJ2284 (96.67%) and LJ2285 (99.35%) and of TTAGGC in LJ2400 (98.11%) and LJ2406
211 (99.73%) (Fig. 1G, Supplemental Fig. S6 and Supplemental Table S9). These findings
212 supported our TRM study employing short-read WGS data and revealed that the TTAGAC
213 telomere had evolved in the family Panagrolaimidae.

214 **TTAGAC-containing units clustered near telomeric regions of TTAGGC-telomere** 215 **isolates**

216 Because most of the nematode species that we analyzed had a canonical Nematoda TRM
217 sequence, TTAGGC, the TTAGAC telomere probably evolved from its ancestral TTAGGC
218 telomere via a single nucleotide mutation of the telomerase RNA component gene. We
219 hypothesized that subtelomeric sequences can provide evidence regarding the evolutionary
220 mechanism because these can carry historical records of telomere dynamics. For example, it
221 has previously been documented that ALT mechanisms have been used to reconstruct
222 subtelomeric and telomeric regions in *C. elegans* and that templates for ALT in subtelomeric
223 regions can be used to repair telomere damage or maintain the integrity of the telomere
224 (Seo et al. 2015; Kim et al. 2019a; Kim et al. 2020; Kim et al. 2021b; Lee et al. 2022b). Thus, we
225 analyzed subtelomeric regions of our four Panagrolaimidae genome assemblies to trace
226 ancestral telomere damage and repair events that could explain how the TTAGAC telomere
227 evolved from the TTAGGC telomere.

228 Most telomeric repeats in telomeric regions (7/10 in LJ2284, 4/8 in LJ2285, 6/8 in LJ2400, and
229 8/14 in LJ2406) were attached to unit clusters containing TTAGGC or TTAGAC (e.g.
230 [TTAGGCTTAATTGC]_n or [TTAGACTTATTCGC]_n) (Fig. 2A and Supplemental Table S10). This
231 finding was striking, as none of the subtelomeric regions of *C. elegans* and *B. xylophilus*
232 exhibited TRM-containing unit clusters directly attached to the telomeric regions (Fig. 2A and
233 Supplemental Table S11). Furthermore, both TTAGGC-containing unit clusters and TTAGAC-
234 containing unit clusters were identified in all genomes of the TTAGGC- and TTAGAC-
235 telomere isolates, with the exception of the LJ2285 genome (TTAGAC telomere), which
236 contained only TTAGAC-containing unit clusters. For LJ2284, LJ2400, and LJ2406, these
237 clusters consisted of repetitive units with length distributions ranging from 14 bp to ~2 kb,
238 and these repetitive units were tandemly repeated on an average of ≥ 19 copies
239 (Supplemental Tables S10 and S12). The LJ2285 genome did not contain such same-length
240 unit clusters; however, it contained sequence clusters consisting of 5–9 copies of units
241 containing TTAGAC and similar sequences (10–74 bp). These TRM-containing unit clusters
242 were not found outside the subtelomeric region in the telomere-containing contigs/scaffolds
243 (see also Supplemental Note describing a contrasting case in *C. elegans*), raising the
244 possibility that these clusters are associated with telomere maintenance in Panagrolaimidae.
245 Notably, we also identified a similar subtelomeric pattern in *Caenorhabditis uteleia*, the
246 noncanonical TTAGGT-TRM species, where TTAGGT- and/or TTAGGC-containing unit clusters
247 were attached to or close to long TTAGGT repeat arrays (Supplemental Fig. S7 and
248 Supplemental Note) (SRA accession: ERR8978452) (The Darwin Tree of Life Project
249 Consortium 2022).

250



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252

253 **Figure 2.** Subtelomere structures and a proposed model to explain clustered blocks of TRMs

254 or TTAGRC-containing unit clusters in the subtelomeric regions. (A) Schematic representation

255 of subtelomere structures in the contigs/scaffolds listed in Supplemental Table S10. We

256 categorized the structure of subtelomeric regions (up to 200 kb from the end of the
257 telomeric contig/scaffold) using the following criteria: (i) whether it has ITSs (shorter red
258 blocks) and/or TTAGRC-containing unit clusters (half-sky blue, half-violet blocks) and (ii)
259 whether ITSs, unit clusters, and telomeric regions were directly attached or separated by
260 other sequences (white, empty blocks). Each horizontal bar represents a subtelomere
261 structure with only a telomeric TTAGRC-containing unit cluster (shown in Structure 4 and 5)
262 and an ITS or a TTAGRC-containing unit cluster closest to the telomere (shown in Structure 2
263 or 3). Each value refers to the number of contigs/scaffolds with the corresponding structure
264 type in each species/isolate. TTAGGC-telomere species and isolates had ITSs that were
265 composed of TTAGGC, rather than TTAGAC, and TTAGAC-telomere species and isolates had
266 only TTAGAC-type ITSs, too. (B) A proposed model for generating subtelomere structures
267 responsible for Structure 5. After telomere damage, HDR mechanisms can exploit homology
268 between shortened telomeric repeats and ITSs at other loci to repair the damaged telomere.
269 A HDR mechanism, break-induced replication (BIR), may replicate sequences near the ITS,
270 creating a new homologous block of the original sequence block. If the original template
271 block exhibits a TTAGRC-containing unit cluster, the cluster would also be replicated. Finally,
272 a new telomere composed of TTAGGC repeats can be replenished by active telomerase.

273

274 In addition to the TRM-containing unit clusters, interstitial telomeric sequences (ITSs) were
275 frequently observed in the subtelomeric regions of the constructed genome assemblies,
276 which were consistent with the ITS enrichment present in *C. elegans* chromosome arms (The
277 *C. elegans* Sequencing Consortium 1998). All subtelomere structures were categorized based

278 on the location of the unit clusters and/or the ITSs in subtelomeric regions (up to 200 kb
279 from the end of the telomeric contig/scaffold) (Supplemental Tables S10 and S11). We
280 annotated a total of 12 subtypes (Supplemental Fig. S8), but for simplicity we merged them
281 into five major subtelomere structures by considering only telomeric TTAGRC-containing unit
282 clusters and ITSs or TTAGRC-containing unit clusters closest to the telomere (Structure 1–5 in
283 Fig. 2A; see Supplemental Fig. S9 for examples of Structure 2–5). Notably, the motif in all ITSs
284 was the same as their TRM. The four genome assemblies exhibited similar patterns. For most
285 of the subtelomere structures in the four genome assemblies, the TRM-containing unit
286 cluster was positioned directly adjacent to the telomeric region. Structure 1, which did not
287 contain any unit cluster or ITS, accounted for fewer than a quarter of all the subtelomere
288 structures (the lengths of telomeres in Structure 1 differ from those of ITSs in *C. elegans* and
289 our four isolates; see Supplemental Note). In contrast, Structure 2–5 had at least one unit
290 cluster or ITS separated from the telomeric region, which may have resulted from ancestral
291 telomere damage and repair processes.

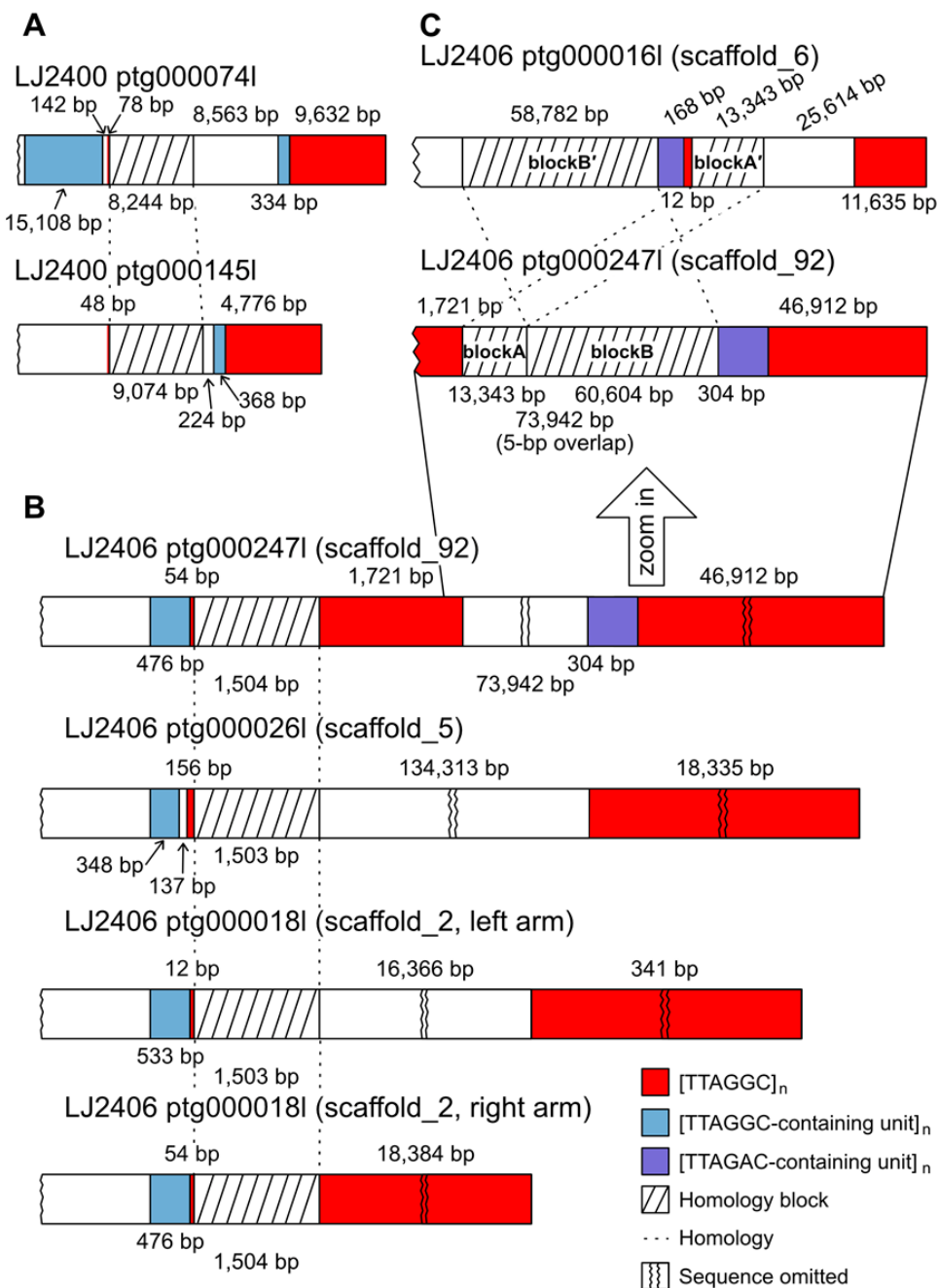
292 **TTAGAC-containing units were probably used to maintain the integrity of TTAGGC**
293 **telomeres**

294 We postulated that the identified subtelomeric clusters of the TTAGRC-containing units and
295 ITSs were evidence of ancestral telomere damage and repair via ALT mechanisms and that if
296 true, this would help understand the role of TTAGRC-containing unit clusters in telomere
297 maintenance. Among Structure 2–5, Structure 5 may be explained by BIR, one of the most
298 well-known ALT mechanisms for telomere repair (Malkova et al. 1996; Bosco and Haber 1998;
299 Kim et al. 2019a; Kim et al. 2020). Telomeres that have been damaged or shortened should

300 be repaired, and BIR may rely on homology between shortened telomeric repeats and ITSs at
301 other loci (Fig. 2B, left side). The replication was then activated, and occasionally, sequences
302 beside the ITS would also be replicated (Fig. 2B, middle). Furthermore, if the original
303 homology block contained a TTAGRC-containing unit cluster, the cluster would also be
304 duplicated simultaneously, resulting in a new subtelomere structure with a homology block
305 interposed between the ITS and the TTAGRC-containing unit cluster. Finally, a new telomere
306 composed of TTAGGC repeats could be replenished by active telomerase (Fig. 2B, right side).
307 We then hypothesized that if Structure 5 cases in our genomes had been generated via BIR,
308 we should be able to identify another sequence block homologous to the interposed
309 sequence between the ITS and the TTAGRC-containing unit cluster.

310 To test this hypothesis, we searched for homology blocks in the sequences between an ITS
311 and a TTAGRC-containing unit cluster from each of three contigs representing Structure 5 in
312 whole genome sequences (ptg000074I and ptg000145I of LJ2400 and ptg000247I of LJ2406)
313 (Fig. 2, Fig. 3, Supplemental Fig. S8, and Table S10). ptg000074I and ptg000145I of LJ2400
314 shared 8-kb and 9-kb homology blocks adjacent to the ITS and had a TTAGGC-containing
315 unit cluster ~9-kb and ~200-bp away from the shared block, respectively (Fig. 3A). These 8-
316 kb and 9-kb homology blocks pointed to the possibility of their replication via BIR using the
317 ITS as homology.

318



319

320

321 **Figure 3.** Traces of telomere damage and repair processes through ALT mechanisms

322 identified in subtelomeric regions of genome assemblies of TTAGGC-telomere isolates. (A–C)

323 Schematic representations of subtelomeres in the genome assemblies of LJ2400 and LJ2406.

324 Hatched boxes indicate homology blocks between two or more subtelomeres, and dashed
325 lines indicate homologous relationships. The red boxes represent telomeric-repeat clusters.
326 Violet and sky blue boxes represent TTAGAC-containing unit clusters and TTAGGC-
327 containing unit clusters, respectively. Not to scale.

328

329 The ptg000247l of LJ2406 had a more evident BIR signature than the ptg000074l and
330 ptg000145l of LJ2400, indicating that the subtelomeric TTAGGC-containing unit cluster was
331 built via the ALT process. The subtelomeric region of ptg000247l of LJ2406 consisted of a
332 TTAGGC-containing unit cluster, a 54-bp ITS, a 1.5-kb sequence block, a 1.7-kb ITS, a 74-kb
333 sequence block, and a TTAGAC-containing unit cluster close to the telomeric region (Fig. 3B).
334 By searching for this complex subtelomeric sequence in the whole genome using BLAST, we
335 determined that the 1.5-kb and 74-kb blocks had homologous sequences in other
336 subtelomeric regions. The 1.5-kb block between the 54-bp ITS and 1.7-kb ITS had almost
337 identical homologous sequences in three different subtelomeric regions. These shared blocks
338 were directly adjacent to short and variable-length ITSs near TTAGGC-containing unit
339 clusters (Fig. 3B). The TTAGGC-containing cluster and ITS may have been utilized for
340 homology to replicate the 1.5-kb homology blocks.

341 The 74-kb block between the 1.7-kb ITS and the TTAGAC-containing unit cluster possessed
342 another trace of HDR and an additional replication of a TTAGGC-containing unit cluster after
343 telomere damage (Fig. 3C). As the 1.7-kb length of the ITS is too long to be created by
344 random mutation, it could represent evidence of ancestral telomere damage and repair. The

345 74-kb block contained at least two distinct homology blocks: the 13-kb blockA and the 61-
346 kb blockB (Fig. 3C). The 13-kb blockA directly linked to the ITS in ptg000247l had an identical
347 blockA' in ptg000016l that was also directly attached to another 180-bp sequence of an ITS
348 and a TTAGAC-containing unit cluster. The 61-kb blockB was homologous but not identical
349 to the 59-kb blockB' located on the opposite side of the 180-bp block of the ITS and the
350 TTAGAC-containing unit cluster in ptg000016l. Of note, the last 5 bp of the 13-kb blockA and
351 the first 5 bp of the 61-kb blockB were identical (i.e. TAAAT). The 61-kb blockB was further
352 divided into four blocks, each of which also exhibited microhomologies with the adjacent
353 blocks (Supplemental Fig. S10 and Supplemental Table S13). These data imply that telomere
354 damage and repair may have occurred in the following order: first, the 13-kb blockA in
355 ptg000247l was replicated by BIR using homology between the 1.7-kb ITS in ptg000247l and
356 the 180-bp block of the ITS and the TTAGAC-containing unit cluster in ptg000016l. Next, the
357 61-kb blockB in ptg000247l was replicated by multiple rounds of microhomology-mediated
358 BIR (MMBIR) and template switching events using microhomology sequences at the end of
359 the blocks (Supplemental Fig. S10 and Supplemental Note).

360 This 59-kb blockB' in ptg000016l was directly attached to the TTAGAC-containing unit
361 cluster, which was duplicated in ptg000247l. Notably, the original TTAGAC-containing unit
362 cluster in ptg000016l was shorter than the duplicated cluster in ptg000247l, implying that the
363 duplicated cluster nearly doubled after MMBIR was completed. The duplicated TTAGAC-
364 containing unit cluster in ptg000247l was directly linked to the TTAGGC telomere, indicating
365 that the duplicated cluster was exposed at the end. These lines of evidence support the
366 notion that even in TTAGGC-telomere species, TTAGAC-containing units could be

367 incorporated to constitute a telomere or at least serve as a DNA template for replenishing
368 TRM repeats by telomerase. This use of TTAGAC-containing units in the TTAGGC-telomere
369 isolate may have contributed to the evolution of the TTAGAC telomere from the TTAGGC
370 telomere.

371 **Telomere-associated protein genes exhibited similar conservation patterns among**
372 **Panagrolaimidae species/isolates**

373 We hypothesized that TTAGAC-containing unit clusters served as a component of partially
374 stable telomere in TTAGGC-telomere species and that TBPs for TTAGGC telomere could have
375 bound TTAGAC repeats and have been conserved in both TTAGGC-telomere and TTAGAC-
376 telomere species. To support this hypothesis, we analyzed protein homologies in
377 Panagrolaimidae with *B. xylophilus* as an outgroup species, in which proteins have been
378 described to maintain or bind telomeres in *C. elegans* (Ahmed and Hodgkin 2000; Hofmann
379 et al. 2002; Im and Lee 2003; Kim et al. 2003; Joeng et al. 2004; Im and Lee 2005; Meier et al.
380 2006; Boerckel et al. 2007; Raices et al. 2008; Meier et al. 2009; Ferreira et al. 2013; Shtessel et
381 al. 2013; Dietz et al. 2021; Yamamoto et al. 2021). We identified 12 proteins that exhibited
382 similar homology patterns between TTAGGC- and TTAGAC-telomere species/isolates
383 (Supplemental Fig. S11). In contrast, four proteins (POT-2, TEBP-2 (also known as DTN-2),
384 HPR-9, and MRT-1) displayed inconsistent conservation patterns. POT-2 was conserved in
385 only two of the three TTAGGC-telomere species/isolates, and TEBP-2 remained in only one of
386 the three TTAGAC-telomere species/isolates. HPR-9 was observed in only one TTAGAC-
387 telomere isolate. MRT-1, required for telomerase activity in *C. elegans* (Meier et al. 2009), was
388 not detected in *B. xylophilus* or in any of the three TTAGAC-telomere species/isolates but

389 was present in all three TTAGGC-telomere species/isolates. However, MRT-1 functional
390 domains were not conserved in the TTAGGC-telomere species/isolates. Nonetheless,
391 homologs of the four TBPs were mostly conserved in all Panagrolaimidae species and
392 isolates (Supplemental Table S14). Despite these variations, TRT-1 was conserved in all
393 species and isolates, suggesting that they may have active telomerase and that changes in its
394 RNA template may have been involved in TRM evolution (Fig. 4; see Discussion).

395

396 **DISCUSSION**

397 TRMs and their protein partners are well conserved and mostly co-evolving because they
398 must be connected to maintain the DNA–protein complex, telomeres (Shakirov et al. 2009;
399 Steinberg-Neifach and Lue 2015; Sepsiova et al. 2016; Červenák et al. 2019). Nevertheless,
400 TRMs have evolved across several taxa (Fulnečková et al. 2013; Garavís et al. 2013; Peska and
401 Garcia 2020; Červenák et al. 2021). In this study, we identified three novel TRMs in Nematoda
402 and confirmed that TTAGAC, one of the three novel TRMs, composes telomeres in a subset
403 of Panagrolaimidae isolates. We also hypothesized that TTAGAC evolved through TTAGAC-
404 containing unit clusters and the robustness of the TTAGGC-suited TBPs to bind tandem
405 arrays of TTAGAC repeats in a canonical TTAGGC-telomere ancestor.

406 The consistent pattern of TRM changes observed in other multicellular organisms supported
407 our hypothesis. First, sequence changes in plant TRMs also exhibit key characteristics (Peska
408 and Garcia 2020). In particular, the basal TRM in plants, TTTAGGG, (*Arabidopsis*-type)
409 (Richards and Ausubel 1988) evolved into _TTAGGG (vertebrate-type) (Weiss and Scherthan

410 2002; Sýkorová et al. 2003; Sýkorová et al. 2006), ITTTAGGG (*Chlamydomonas*-type)
411 (Fulnečková et al. 2012), TTCAGG (*Genlisea*-type) (Tran et al. 2015), ITTCAGG (*Genlisea*-
412 type) (Tran et al. 2015), ITTTAGG (*Klebsormidium*-type) (Fulnečková et al. 2013), and
413 TTTTTAGGG (*Cestrum*-type) (Peška et al. 2015). Briefly, these variant TRMs exhibited
414 alterations in the lengths of the T and G arms or changes in pyrimidine (T to C) nucleotides
415 close to the middle A. Pyrimidine nucleotide conversion, rather than changes between
416 pyrimidine and purine nucleotides and vice versa, has also been observed in insects in which
417 TTAGG changed to TTCAGG (Mravinac et al. 2011). Similarly, in the current study, TTAGGC
418 changed to TTAGGI (pyrimidine conversion) and TTAGAC (purine conversion) in nematodes.
419 Considering pyrimidines (T and C) have comparable base sizes and purines (G and A) are of
420 similar sizes, we propose that conversions between pyrimidines or between purines do not
421 sterically hinder TBP binding, allowing pre-existing TBPs to attach to the modified TRM.
422 Furthermore, in yeasts and plants, TBPs are sufficiently robust to bind to various types of
423 TRM despite several base pair changes (Rotková et al. 2004; Fajkus et al. 2005; Kramara et al.
424 2010; Visacka et al. 2012; Červenák et al. 2019; Tomáška et al. 2019; Červenák et al. 2021). In
425 yeast, a TBP binds at least six different types of TRMs, implying binding robustness of TBPs
426 during TRM evolution (Červenák et al. 2019). Here we suggest that this idea could be further
427 supported by TTAGAC-containing unit clusters in TTAGGC-telomere species.

428 It is still unclear whether TTAGGC-binding TBPs also bind to TTAGAC in TTAGGC-telomere
429 Panagrolaimidae species/isolates. Traces of ALT action identified in our genome assemblies
430 revealed that TTAGAC-containing units were used to repair telomere damage in TTAGGC-
431 telomere isolates. Two TTAGGC-telomere isolates and one TTAGAC-telomere isolate

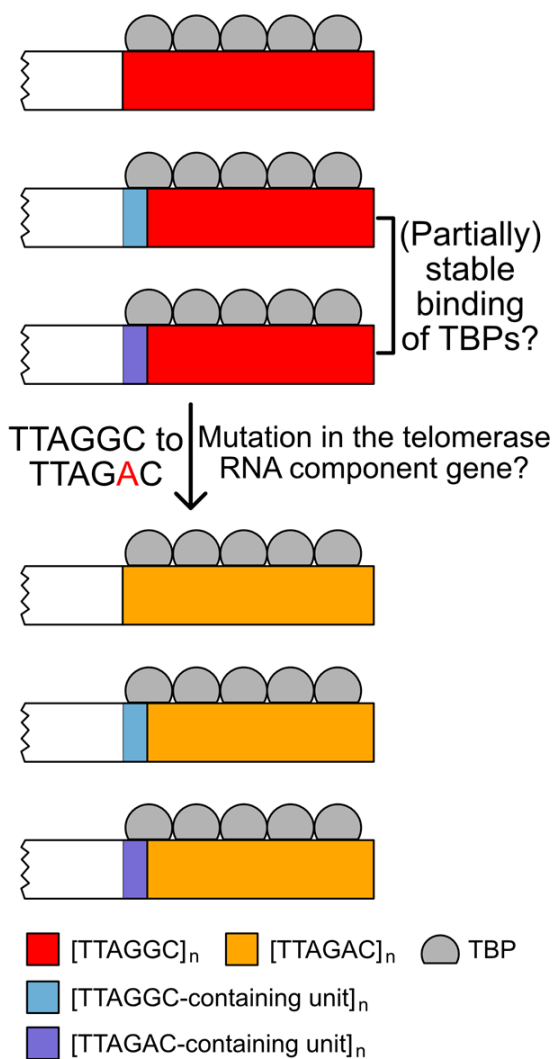
432 examined harbored both clusters of consecutive TTAGGC- and TTAGAC-containing units.
433 Some clusters were attached directly or were adjacent to the telomeres. Specifically, the
434 subtelomeric sequences of ptg000247l in LJ2406, a TTAGGC-telomere isolate, showed
435 evidence of direct usage of a TTAGAC-containing unit for the repair of a damaged telomere.
436 In the vicinity of a damaged and shortened telomere, there were homologous sequence
437 blocks and a TTAGAC-containing unit cluster, which were probably replicated by BIR and
438 MMBIR, and the TTAGAC-containing unit cluster was directly adjacent to its telomeric
439 TTAGGC cluster. Thus, at least over a brief period of time, the TTAGAC-containing unit cluster
440 was exposed at the end of the chromosome before the TTAGGC repeats were replenished.
441 Furthermore, the TTAGAC-containing unit cluster was not only duplicated but also elongated
442 at the end because it was nearly twice as long as its initial cluster template in a separate
443 subtelomeric region. This cluster might have been elongated via replication slippage based
444 on its repetitive nature or via ALT mechanisms that can replicate TRM-containing units at the
445 end of the chromosome. We could not determine the specific mechanisms involved in the
446 elongation of the TTAGAC-containing unit cluster. If the elongation was achieved via ALT
447 mechanisms, this suggests that the TTAGAC-containing unit cluster exhibits its own
448 replication capability in the telomeric region to maintain the telomere.
449 If the TTAGAC-containing unit cluster acted as a component of the telomere, the TTAGGC-
450 suited TBPs would bind to the telomere; otherwise, the end would be recognized as a DNA
451 damage site. This binding robustness of TBPs, if it exists as reported in fungi, may explain the
452 subtelomere structure and TRM evolution. This robustness could be achieved via two
453 potential ways: (1) the TTAGGC-suited TBPs also exhibited sufficient binding affinity for

454 TTAGAC, such that TTAGAC-unit clusters could be utilized for repairing telomere damage.
455 This binding affinity stabilized the TTAGAC-containing unit–TBP complex as a telomere. (2)
456 TBPs exhibited a weak binding affinity to TTAGAC and formed a partially stable telomere
457 complex, and the affinity strengthened through frequent use of TTAGAC-containing unit
458 clusters to repair telomere damage and ensure fitness. In both cases, TTAGGC-suited TBPs
459 could also stabilize TTAGAC telomeres, which thus facilitated the conversion from TTAGGC
460 telomeres to TTAGAC telomeres.

461 Our results suggest a plausible evolutionary model for the TRM conversion in the family
462 Panagrolaimidae. First, ancestral TTAGGC-telomere species may have utilized various ALT
463 mechanisms to counteract telomere damage when active telomerase cannot access the
464 damaged telomere (Fig. 4). Replication of TTAGGC-containing unit clusters could represent
465 one such ALT mechanism and would have been used to repair damaged telomeres.
466 Consequently, active telomerase may use unit clusters as a starting template to fully
467 replenish the partially repaired telomere. Because TBPs can fully or partially stabilize
468 telomere complexes consisting of TTAGAC-containing unit clusters, the evolution of the TRM
469 conversion to TTAGAC had a greater fitness than other TTAGGC variants. We could not
470 identify the telomerase RNA component gene in Panagrolaimidae. However, conservation of
471 TRT-1 and highly accurate replication of TRMs at the telomere probably support the
472 presence of an active telomerase in these species/isolates (Fig. 1G and Supplemental Fig. S6
473 and S11). TRM would have converted to TTAGAC via mutation in the telomerase RNA
474 component gene, leading to TTAGAC telomeres, although it remains unclear whether this
475 change involved a single mutation in the gene or coexistence of the original and mutated

476 genes. This transition may have occurred long ago, since TTAGAC- and TTAGGC-telomere
 477 isolates had only TTAGAC- and TTAGGC-ITSs, respectively. Moreover, both telomeres and
 478 ITSs consisted of the same TRM, and sequences of their ITSs were not much degenerated,
 479 indicating that these ITSs resulted from recent, and probably frequent, telomere damage
 480 events in these isolates.

481



482

483

484 **Figure 4.** A model for TRM evolution in Panagrolaimidae. If TBPs with affinity for TTAGGC
485 telomeres could bind to any of the TTAGGC telomere, TTAGGC-containing unit clusters, or
486 TTAGAC-containing unit clusters, they were able to construct a fully or partially stable
487 telomere. This binding robustness of TBPs may have facilitated the TRM conversion from
488 TTAGGC to TTAGAC. Even after TRM changed via mutation of the telomerase RNA
489 component gene, if it occurred, TBPs could still bind to altered telomeric repeats, which
490 possibly contributed to the evolution of a novel TRM, TTAGAC, in the Panagrolaimidae
491 family.

492

493 It is unclear whether TTAGGC-suited TBPs could bind to the novel telomeric sequence;
494 however, if this were possible, the TTAGAC cluster–TBP complex would be partially stabilized,
495 preventing the new telomere from being recognized as a DNA damage site. This hypothesis
496 can be evaluated directly by inducing a mutation in the telomerase RNA component gene
497 and then measuring the robustness of TBP in terms of sequence specificity. Unfortunately,
498 we could not identify the telomerase RNA component gene for these isolates, as the gene
499 has not been identified even in *C. elegans*. Further studies are required to support our
500 hypothesis. Nonetheless, we identified TTAGGC- and/or TTAGGT-containing unit clusters in
501 putative subtelomeric regions of *Caenorhabditis ueteleia*, which suggests that TRM-containing
502 unit clusters are associated with telomere maintenance and that their usage in telomere
503 maintenance contributes to TRM evolution (see also Supplemental Note).

504 Our results describe the independent evolution of three novel TRMs in Nematoda. We found
505 that an ALT-mediated mechanism may have been used to repair ancestral telomere damage
506 in Panagrolaimidae nematodes. Further, we hypothesized that the use of TTAGAC-containing
507 units and the robustness of TBPs facilitated the evolution of the novel TRM. As the
508 robustness of the TBPs may depend on the same-sized bases between canonical and novel
509 TRMs, the evolutionary path we propose for Panagrolaimidae can also be applied to plants,
510 insects, or other nematodes where mutations occur only between pyrimidine nucleotides or
511 between purine nucleotides. Although our study cannot explain all TRM changes in
512 Nematoda, it provides new insight into telomere evolution through ALT and the robustness
513 of TBPs.

514

515 **METHODS**

516 **Worm sampling and culture**

517 Nematodes were collected from rotten fruits in the Republic of Korea (see Supplemental
518 Table S5 for more information). They were grown in plates containing nematode growth
519 medium seeded with *Escherichia coli* strain OP50 and contaminated by their natural
520 microbes.

521 **DNA/RNA extraction and sequencing**

522 Mixed-stage worms were lysed, and genomic DNA was extracted using the Gentra Puregene
523 Cell and Tissue Kit (QIAGEN) to obtain WGS data for Panagrolaimidae isolates, LJ2281,

524 LJ2284, LJ2285, LJ2400, LJ2402, and LJ2406. Macrogen (South Korea,
525 <https://www.macrogen.com/en/main>) prepared DNA sequencing libraries using TruSeq Nano
526 DNA and performed 151-bp paired-end DNA sequencing on the Illumina NovaSeq 6000
527 platform. One adult female worm was lysed, and its genomic DNA was amplified in the same
528 0.2-mL PCR tube using the REPLI-g single-cell WGA kit (QIAGEN) to obtain single-worm WGS
529 data from LJ2050, LJ2051, LJ2060, LJ2070, LJ2072, LJ2401, LJ2411, and LJ2417. Paired-end
530 DNA sequencing was performed using the Illumina NovaSeq 6000 platform by Theragen Bio
531 (South Korea, <https://www.theragenbio.com/en/>).

532 Long-read DNA sequencing and short-read RNA sequencing of LJ2284, LJ2285, LJ2400, and
533 LJ2406 were conducted as described by Kim et al. (Kim et al. 2019a) and Lee et al. (Lee et al.
534 2022a). In summary, genomic DNA was extracted from mixed-stage worms using
535 phenol/chloroform/isoamyl alcohol (25:24:1) and sequenced using the HiFi mode of the
536 PacBio Sequel II platform by Macrogen. RNA was extracted from mixed-stage worms using
537 the TRIzol method. RNA sequencing libraries were prepared using TruSeq Nano DNA and
538 sequenced by Macrogen using the Illumina NovaSeq 6000 platform with paired-end reads.

539 **Preparing publicly available WGS data**

540 We included Nematoda species in our study, whose genome assembly data were available in
541 the GenBank database and short-read sequencing data were available in the NCBI Sequence
542 Read Archive (SRA) (RRID:SCR_004891). The following criteria were used to filter the short-
543 read sequencing data: Instrument, *Illumina* (if HiSeq data were available, we used HiSeq;
544 otherwise, MiSeq, NextSeq, and Genome Analyzer II data were selected); Source, *Genomic*;

545 Layout, *PAIRED*; Number of Bases, >5 Gb; Spot number, >5 million; Read length, >70 bp.
546 Nematode clades were classified according to the phylogenetic tree (Smythe et al. 2019). Any
547 genus that was not included in the phylogenetic tree was excluded. WGS data for *Enoplus*
548 *brevis*, which lacked genome assembly information in the GenBank database, and WGS data
549 for *Panagrellus redivivus* (Srinivasan et al. 2013) that was supplied by Dr. P. W. Sternberg
550 (now it can be accessible via NCBI SRA under accession number SRR25684730) were added
551 to the filtered datasets. Detailed accession information is available in Supplemental Tables S1
552 and S2.

553 **Identifying TRMs using short-read WGS data**

554 To normalize the public WGS data, we trimmed all reads to 60 bp and used only 5 million
555 sub-sampled reads from the R1 files using Seqtk (<https://github.com/lh3/seqtk>) (version 1.3-
556 r106; `seqtk sample -s 11 - 5000000`). For each dataset, 23-mers were counted using Kounta
557 (<https://github.com/tseemann/kounta>) (version 0.2.3; `kounta --kmer 23 --out`). Only tandemly
558 repeated sequences with unit lengths of 5–7 bp and counts >99 were used to identify
559 putative TRMs that were most frequent and most similar to the canonical TTAGGC sequence.
560 Methods used to further validate the novel putative TRMs are described in Supplemental
561 Methods. We averaged the counts of the 23-mers that contained the corresponding TRM
562 concatemers to compare the number of TRMs across species. For the Panagrolaimidae
563 isolates, we used 20 million sub-sampled reads and all 23-mers, as telomeric repeats were
564 not evenly amplified during whole-genome amplification.

565 **Genome assembly**

566 HiFi reads were *de novo* assembled using Hifiasm (Cheng et al. 2021) (version 0.13-r308;
567 *hifiasm -l0*). Contigs that were potentially contaminated with bacterial sequences were
568 removed as described by Kim et al. (Kim et al. 2019a). Scaffolding and visualization based on
569 the Hi-C data are described in Supplemental Methods. The completeness of the genome
570 assembly was evaluated using BUSCO (Simão et al. 2015) (version 4.0.6; *busco -m genome -l*
571 *nematoda_odb10*) with the nematoda dataset in the OrthoDB release 10. Isolate-specific
572 repetitive sequences were identified using BuildDatabase (Flynn et al. 2020) (version 2.0.1;
573 default options) and RepeatModeler (Flynn et al. 2020) (version 2.0.1; *RepeatModeler -*
574 *database -LTRStruct*). Isolate-specific and known metazoan-repetitive sequences were
575 masked using RepeatMasker (Chen 2004) (version 4.1.0; *RepeatMasker -lib -s* for species-
576 specific repeats and *RepeatMasker -species metazoa -s* for metazoan repeats). We mapped
577 the RNA-seq reads to the repeat-masked genome using HISAT2 (Kim et al. 2019b) (version
578 2.2.1; *hisat2-build* for genome indexing and *hisat2* with default options to map the RNA-seq
579 reads to the corresponding genome). RNA-mapping data was used to annotate genes using
580 BRAKER (Stanke et al. 2006; Stanke et al. 2008; Li et al. 2009; Barnett et al. 2011; Lomsadze et
581 al. 2014; Buchfink et al. 2015; Hoff et al. 2016; Hoff et al. 2019; Bruna et al. 2021) (version
582 2.1.5; *braker.pl --genome -bam --softmasking*).

583 **Preparation of 18S ribosomal DNA sequences to generate the Panagrolaimidae** 584 **phylogenetic tree**

585 For *Panagrolaimus* sp. PS1159, *Panagrolaimus davidi*, *Panagrellus redivivus*, and
586 *Bursaphelenchus xylophilus*, we used publicly available 18S rDNA sequences (GenBank
587 accessions: U81579.1, AJ567385.1, AF083007.1, and KJ636306.1, respectively). For the HiFi-

588 based *de novo* genome assemblies, we extracted 18S rDNA sequences by searching known
589 nematode 18S rDNA PCR primers (nSSU_F_04: 5'-GCTTGTCTCAAAGATTAAGCC-3' (Blaxter et
590 al. 1998) and nSSU_R_82: 5'-TGATCCTTCTGCAGGTTACCTAC-3' (Medlin et al. 1988)) in
591 genome assemblies using BLAST+ (Camacho et al. 2009) (version 2.7.1; *makeblastdb -*
592 *input_type fasta -dbtype nucl* and *blastn -task blastn-short -outfmt 6*).

593 For the other TTAGAC-TRM isolates used in this study, we mapped their short-read DNA
594 sequencing data to the LJ2285 genome assembly using BWA-MEM (version 0.7.17; *bwa*
595 *mem*, default option), and the 18S rDNA sequence variation of each isolate were identified
596 using BCFtools (Li 2011a) (version 1.13; *bcftools mpileup -Ou -f | bcftools call -Ou -mv |*
597 *bcftools norm -f -Oz -o*). The indexed output VCF files were obtained using Tabix (Li 2011b)
598 (version 1.13; default option), and the LJ2285 18S rDNA sequence was replaced with their
599 variants of each isolate using SAMtools (Li et al. 2009) (version 1.13) and BCFtools (*samtools*
600 *faidx -r | bcftools consensus -o*). For the other TTAGGC-TRM isolates, we used the LJ2400
601 genome assembly as a reference and repeated the procedure described above. All 18S rDNA
602 sequences of Panagrolaimidae obtained in this study are listed in Supplemental Table S6. We
603 generated an alignment file using all 18S rDNA sequences as input for Clustal Omega
604 (<https://www.ebi.ac.uk/Tools/msa/clustalo/>, RRID:SCR_001591, (Sievers and Higgins 2021))
605 (Sequence Type: DNA; Output Alignment Format: PHYLIP). We used this alignment as an
606 input to RAxML (Stamatakis 2014) (version 8.2.12) using raxmlGUI 2.0 (Edler et al. 2021)
607 (version 2.0.10; options: GTRGAMMA and ML+rapid bootstrap with 1000 replications) to
608 infer the phylogenetic relationships, which were visualized using Dendroscope (Huson and
609 Scornavacca 2012) (version 3.8.4).

610 **Telomere and subtelomere structure in the genome assemblies**

611 We selected candidate telomere-containing contigs using two tandemly repeated copies of
612 TTAGRC that appeared ≥ 3 times in the 600-bp region at each end of the contig. We
613 manually validated these telomeric regions using the following criteria: TRM cluster length
614 ≥ 500 bp (the longest ITS length in *C. elegans*) (Yoshimura et al. 2019) or TRM cluster still
615 located at the end after scaffolding (only for LJ2284 and LJ2406). We analyzed whether each
616 telomere-containing contig/scaffold had clusters with ≥ 6 copies of TRMs or whether
617 TTAGRC-containing units repeated tandemly in its subtelomeric region (up to 200 kb from
618 the end of the contig/scaffold). For LJ2285, we considered sequences consisting of 5–9
619 copies of different units that contained TTAGRC and similar sequences as unit clusters
620 because LJ2285 did not contain clusters composed of the same units. Subsequently, we
621 investigated whether the unit cluster (containing ≥ 6 copies of units, the same TTAGRC and
622 identity $\geq 85\%$ of units) existed outside the subtelomeric region of telomere-containing
623 contigs/scaffolds using BLAST+ (Camacho et al. 2009) (version 2.12.0; *makeblastdb -*
624 *input_type fasta -dbtype nucl; blastn -task blastn-short -outfmt 6* for sequences < 30 bp and
625 *blastn -task megablast -outfmt* for sequences ≥ 30 bp). Unit sequences of LJ2284, LJ2400, and
626 LJ2406 and unit cluster sequences in LJ2285 were denoted starting with TTAGRC
627 (Supplemental Table S12). The BIR traces were analyzed by searching for homologous
628 sequences between ITSs, TTAGRC-containing unit clusters, or telomeric repeats against their
629 corresponding genome assembly using BLAST+ (Camacho et al. 2009) (version 2.7.1;
630 *makeblastdb -input_type fasta -dbtype nucl and blastn -task megablast -outfmt 6*).

631 **Conservation analysis of telomere-associated proteins**

632 Protein FASTA sequences were downloaded from WormBase for *C. elegans* (Release WS281)
633 and WormBase ParaSite (Howe et al. 2017) for *Panagrolaimus* sp. PS1159, *Panagrolaimus*
634 *dauidi*, *Panagrellus redivivus*, and *B. xylophilus* (Release WBPS15). Protein sequences for
635 *Panagrolaimus* sp. PS1579 and *Panagrolaimus* sp. ES5 were not publicly available. For the
636 four Panagrolaimidae isolates (LJ2284, LJ2285, LJ2400, and LJ2406), we used protein FASTA
637 sequences obtained through gene annotation using BRAKER. For the non-*C. elegans*
638 nematodes, we searched for protein sequences that were conserved in *C. elegans* using
639 DIAMOND (Buchfink et al. 2021) (version 2.0.11; *diamond blastp -d -q -o --threads 20 --very-*
640 *sensitive* and *diamond blastp -d -q -o --threads 20 --ultra-sensitive*) to characterize telomere-
641 associated protein sequences that included TRT-1, POT-1, POT-2, POT-3, MRT-1, MRT-2,
642 TEBP-1 (also known as DTN-1), TEBP-2 (also known as DTN-2), HPR-9, HPR-17, HUS-1, SUN-
643 1, CEH-37, HMG-5, HRP-1, and PLP-1. We filtered the nematode's highest bit score protein
644 sequence for each *C. elegans* telomere-associated protein and identified the conserved
645 domains using NCBI CD-search (Marchler-Bauer and Bryant 2004; Lu et al. 2020) (CDD
646 database, version 3.19).

647

648 DATA ACCESS

649 Raw sequencing data and genome assemblies have been submitted in the NCBI BioProject
650 database (<https://www.ncbi.nlm.nih.gov/bioproject>) under the accession number
651 PRJNA845886 and in the Korean Nucleotide Archive (KoNA, <https://kobic.re.kr/kona>) under
652 the BioProject accession number KAP220348 (sequencing data only).

653

654 **COMPETING INTEREST STATEMENT**

655 None declared.

656

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667

668 **AUTHOR CONTRIBUTIONS**

669 Jiseon Lim: Conceptualization, Methodology, Formal Analysis, Investigation, Writing-Original
670 Draft, Writing-Review & Editing. Wonjoo Kim: Investigation. Jun Kim: Conceptualization,
671 Methodology, Investigation, Writing-Original Draft, Writing-Review & Editing. Junho Lee:
672 Conceptualization, Writing-Review & Editing, Funding Acquisition, Supervision.

673

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