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Running title: Unique compositional anomaly in pandoraviruses

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Abstract

The Pandoraviridae is a rapidly growing family of giant viruses, all of which have been isolated using laboratory strains of Acanthamoeba. The genomes of ten distinct strains have been fully characterized, reaching up to 2.5 Mb in size. These double-stranded DNA genomes encode the largest of all known viral proteomes and are propagated in oblate virions that are among the largest ever-described (1.2 µm long and 0.5 µm wide). The evolutionary origin of these atypical viruses is the object of numerous speculations. Applying the Chaos Game Representation to the pandoravirus genome sequences, we discovered that the tetranucleotide (4-mer) "AGCT" is totally absent from the genomes of 2 strains (*P. dulcis* and *P. quercus*) and strongly underrepresented in others. Given the amazingly low probability of such an observation in the corresponding randomized sequences, we investigated its biological significance through a comprehensive study of the 4-mer compositions of all viral genomes. Our results indicate that "AGCT" was specifically eliminated during the evolution of the Pandoraviridae and that none of the previously proposed host-virus antagonistic relationships could explain this phenomenon. Unlike the three other families of giant viruses (Mimiviridae, Pithoviridae, Molliviridae) infecting the same Acanthamoeba host, the pandoraviruses exhibit a puzzling genomic anomaly suggesting a highly specific DNA editing in response to a new kind of strong evolutionary pressure.

Importance

The recent years have seen the discovery of several families of giant DNA viruses all infecting the ubiquitous amoebozoa of the genus Acanthamoeba. With dsDNA genomes reaching 2.5 Mb in length packaged in oblate particles the size of a bacterium, the

42 pandoraviruses are the most complex and largest viruses known as of today. In addition
43 to their spectacular dimensions, the pandoraviruses encode the largest proportion of
44 proteins without homolog in other organisms which are thought to result from a *de novo*
45 gene creation process. While using comparative genomics to investigate the evolutionary
46 forces responsible for the emergence of such an unusual giant virus family, we discovered
47 a unique bias in the tetranucleotide composition of the pandoravirus genomes that can
48 only result from an undescribed evolutionary process not encountered in any other
49 microorganism.

50 ## Introduction

51 The Pandoraviruses are among the growing number of families of environmental
52 giant DNA viruses infecting protozoans and isolated using the laboratory host
53 Acanthamoeba (Protozoa/Lobosa/Ameobida/ Acanthamoebidae/ Acanthamoeba) [1-4]. As
54 of today, they exhibit the largest fully characterized viral genomes, made of linear dsDNA
55 molecules from 1.9 to 2.5 Mb in size, predicted to encode up to 2500 proteins[1-3]. After
56 their internalization by phagocytosis, these viruses multiply in their amoebal host
57 through a lytic cycle lasting about 12 hours, ending with the production of hundreds of
58 giant amphora-shaped particles (1.2 µm long and 0.5 µm wide)[1-3]. The phylogenetic
59 structure of the Pandoraviridae family exhibits two separate clusters referred to as A-
60 and B- clades[2,3] (Fig. 1). Despite this clear phylogenetic signal (computed using a core set
61 of 455 orthologous proteins), strains belonging to clade A or B did not exhibit noticeable
62 differences in terms of virion morphology, infectious cycle, host range, or global genome
63 structure and statistics (e.g. nucleotide composition, gene number, gene density)[1-3].

64 In addition to their unusual virion morphology and gigantic genomes, the pandoraviruses
65 exhibit other unique features such as an unmatched proportion (>90%) of genes coding
66 for proteins without any database homologs (ORFans) outside of the Pandoraviridae
67 family, and strain-specific genes contributing to an unlimited pan-genome[1-3]. These
68 features, confirmed by the analysis of additional strains[5], led us to suggest that a process
69 of *de novo* and *in situ* gene creation might be at work in pandoraviruses[2, 3]. Following this
70 history of unexpected findings, we thought that further analyses of the Pandoraviridae
71 might reveal additional surprises.

While searching for hidden genomic patterns eventually linked to evolutionary processes unique to the pandoraviruses, we used a Chaos Game graphical representation of their genome sequences[6-7]. This method converts long one-dimensional DNA sequence into a fractal-like image, through which a human observer may detect specific patterns. This representation illustrates in a holistic manner the frequencies of all oligonucleotides of arbitrary length k (k-mers) in a given DNA sequence. Using this approach led us to discover that the 4-mer "AGCT" was uniquely absent from the genome of *Pandoravirus dulcis*, providing the starting point of the present study (Fig.2).

Results

The absence of any given 4-mer in a long random DNA sequence is highly improbable

After detecting the absence of the "AGCT" word in the Chaos Game graphical representation of the *P. dulcis* genome, we computed the number of occurrence of all 4-mers in the ten available Pandoravirus genome sequences using direct counting[8]. This revealed that "AGCT" was also absent from the genome of *P. quercus*. Notice that although these strains belong to the same A-clade, their genome sequences are nevertheless far from identical (their orthologous coding-regions share 72% nucleotide identity on average), hence the common missing "AGCT" is not a mere consequence of their sequence similarity.

Such a plain finding might not sound very interesting, until one realizes to what extent not encountering a single occurrence of "AGCT" in DNA sequences respectively 1.908.524 bp

(*P. dulcis*) and 2.077.288 bp (*P. quercus*) is unlikely, as shown below, using increasingly sophisticated computations.

In the simplest case, let us first consider a random DNA sequence with equal proportions of the four nucleotides (%A=%T=%C=%G=25%). Since there are 256 distinct 4-mers, the probability for each of them to occur at a given position in an increasingly long sequence tends to $p_{AGCT} = {}^{1}/_{256}$. In a random sequence of approximately 2 Mbp, one thus expects an average of about 7800 occurrences for each distinct 4-mers. This already suggests how unlikely it is for one of them to be absent.

To estimate the order of magnitude of such probability, the DNA sequence is seen as consisting of 4 sets of non-overlapping 4-mers collected according to 4 different "reading frames" (e.g. 4-mers 1-4, 5-8, 9-12, …, etc, for frame 1). The different reading frames thus correspond to approximately 500,000 positions each.

At each of these position, the probability for "AGCT" not to occur is $q_{AGCT} = {}^{255}/_{256}$. For one reading frame, this probability becomes approximately

$$Q_{AGCT} = \left({}^{255}/_{256}\right)^{500,000} \cong 1.2\ 10^{-850} \qquad (1)$$

and:

$$4 \times Q_{AGCT} \cong 5\ 10^{-850} \qquad (2)$$

for the 4 reading frames (assuming them to be independent for the sake of simplicity).

Such a value is smaller than any that could be computed in reference to a physical process. For instance, one second approximately corresponds to 2 10^{-18} of the age of the universe.

The above probability should actually be corrected to account for the fact that we did not specifically search for "AGCT" while analyzing the viral genome. Any missing 4-mer would have raised the same interest. A Bonferroni correction should then be applied to compensate for the multiple testing of 256 different 4-mers. However, the probability of not finding any 4-mer, Q_{any} , remains an incommensurably small number.

$$Q_{any} \cong 256 \times 5\ 10^{-850} \cong 1.3\ 10^{-847} \qquad (3)$$

We may further argue that this event was bound to occur in at least one genome given the huge amount of DNA sequence that is now available, for instance in Genbank. The calculation runs as follows; The april 2019 release of Genbank contains about 3.2 10^{11}bp. Assuming that all Genbank entries are 2 Mb-long sequences, this would correspond to 1.6 10^5 theoretical pandoravirus genomes. The order of magnitude of the probability of observing one of them missing any of the 4-mers remains amazingly small at about

$$Q_{any/Genbank} \cong 1.6\ 10^5 \times Q_{any} \cong 2.1\ 10^{-842} \qquad (4)$$

Finally, one may want to make a final adjustment by taking into account that the *P. dulcis* genome is 64% G+C rich. This slightly changes the probability of random occurrence of "AGCT" from $p_{AGCT} = {}^{1}/_{256} = 0.00391$ to

$$p_{AGCT} = (0.18)^2 \times (0.32)^2 = 3.31\ 10^{-3} \qquad (5)$$

then

$$4 \times Q_{AGCT} = (1 - p_{AGCT})^{500,000} \cong 8.9\ 10^{-719} \qquad (6)$$

Using the same Bonferroni correction as above lead to the final conservative estimate:

$$Q_{any/Genbank} < 4\ 10^{-711} \qquad (7)$$

still an incommensurably small probability (e.g. the same as not getting a single head in 2360 tosses of a fair coin).

As the above computation remains an approximation (neglecting the overlap of neighboring 4-mers), we estimated how unlikely it is that any 4-mer would be missing from large DNA sequences by a different approach. We computer generated a large number of random sequences of increasing sizes and recorded the threshold at which point none of the 4-mers is missing. Fig. 3 displays the results of such computer experiment. It shows how fast the probability of any 4-mer missing is decreasing with the random sequence size. In this experiment, we found that the proportion of sequences larger than 10,000 bp missing anyone of the 256 4-mers was less than 1/10,000.

Caveat: randomized sequences exhibit strongly unnatural 4-mer distributions

The above results already suggested that it is impossible for the *P. dulcis* and *P. quercus* genomes to be missing "AGCT" solely by chance without invoking a biological constraint. However, this conclusion rests on the assumption that the randomization process suitably modeled these genomes. However, the frequency distribution of the various 4-mers found in the actual *P. dulcis* genome (and of other pandoraviruses) and the one computed from its randomized sequence are strongly different (Fig. 4). While the natural sequence consist of 4-mers occurring at frequencies distributed along a large and rather continuous interval, the randomized sequence exhibits 4-mers occurring around 5 narrow peaks of frequencies with none in between. As expected from a good quality randomization, these peaks correspond to the frequencies of the five types of 4-mers: those consisting of only A or T at the lower end, those consisting of only G or C at the

158 higher end, and those consisting of (A or T)/(G or C) in proportions 1/3, 2/2, and 3,1 in
159 between. The more continuous and spread out natural distribution is the testimony of
160 multiple evolutionary constraints, most of them unknown, that have resulted in a distinct
161 4-mer usage, like a dialect or a language tic inherited from past generations[9].

162 First, notice that the missing "AGCT" does not correspond to the 4-mer type with the
163 lowest expected frequency (but the middle one). Second, it is clear that the above
164 probability calculations based on such distorted model of the natural sequence, cannot
165 be used as a reliable estimate of statistical significance. This problem is similar to the one
166 encountered when trying to evaluate the quality of local sequence alignments in similarity
167 searches[10, 11].

168 We can mitigate the effect of the above stringent randomization (only preserving the
169 original nucleotide composition) by using the *P. dulcis* and *P. quercus* actual genome
170 sequences to evaluate to what extent the absence of "AGCT" might be the mere
171 statistical consequence of the frequency of its constituent 3-mers: AGC and GCT.

172 As shown in Table 1, AGC and GCT are not among the least frequent 3-mers found in the
173 *P. dulcis* or *P. quercus* genomes. As the theoretical average is 1/64 (≈ 0.0156), their
174 proportions range from 0.0156 to 0.0097 within the coding and non-coding regions of the
175 genomes. On one given strand, AGC and GCT also do not strongly segregate from each
176 other's in coding *versus* intergenic regions (Table 1). By combining the AGC 3-mer
177 frequency with that of the single nucleotide T ($p_{(t)}$ =0.182 for *P. dulcis*, $p_{(t)}$ =0.196 for *P.*
178 *quercus*), the expected number of "AGCT" per strand is 4286 for *P. dulcis* and 4898 for *P.*
179 *quercus*, while none is observed. Such stark contrast between expected and observed
180 values is unique to the "AGCT" 4-mer. By comparison, the palindromic "ACGT" 4-mer

181 (with an identical composition) exhibits a statistical behavior (Table 1, bottom lines) much
182 closer to the 3-mer-dependent random sequence model.

183

184 ***No 4-mer is missing from the largest actual viral genomes***

185 As vividly illustrated in Fig. 4, the 4-mer distributions in randomized sequences
186 strongly depart from that in natural genomes. We thus analyzed all complete genome
187 sequences available in the viral section of Genbank[12], to investigate to what extent the
188 absence of a given 4-mer was exceptional for genomes in the size range corresponding to
189 Pandoraviruses.

190 We found that the next largest viral genomes missing a 4-mers were those of five phages
191 infecting enterobacteria, with unusual genome sizes in the 345kb-359kb range[13-16]. Except
192 for *P. dulcis* and *P. quercus*, none of the 26 largest publicly available viral genomes
193 (including 25 large/giant eukaryotic viruses, and phage G) [12] were missing a 4-mer (Fig. 5).
194 Thus, even by comparison with natural sequences, *P. dulcis* and *P. quercus* appear
195 exceptional.

196 We noticed that the five large enterobacteria-infecting phages pointed out by our
197 analysis, were all missing the same "GCGC" 4-mer although they exhibit divergent
198 genomic sequences and were isolated from different hosts[13-16]. This palindromic 4-mer
199 might be the target of isoschizomeric restriction endonucleases functionally homologous
200 to HhaI found in *Haemophilus haemolyticus*, a Gammaproteobacteria. Many of them
201 have been described (see https://enzymefinder.neb.com). We will return to the
202 hypothesis that some 4-mers might be missing in response to a host or viral defense
203 mechanism[17] in the discussion section.

204

205 **The anomalous distribution of "AGCT" correlates with the Pandoraviridae phylogenetic**
206 **structure**

207 The absence of "AGCT" in *P. dulcis* and *P. quercus* genomes becomes even more
208 intriguing when put in the context of the phylogenetic structure of the whole
209 pandoravirus family. As shown in Fig. 1, the Pandoraviridae neatly cluster into two
210 separate clades. For well-conserved proteins (such as the DNA polB), the percentage of
211 identical residues between intra-clade orthologs is in the 82% to 90% range, and in the
212 72% to 76% range between the two clades. The corresponding genome sequences are
213 thus far from being identical (and only partially collinear) within each clade. It is thus
214 quite remarkable that the "AGCT" count exhibits a consistent trend to be very low in A-
215 clade members, and at least 10 times higher in B-clade strains. Such a contrast was strong
216 enough to pre-classify three unpublished isolates prior to complete genome assembly and
217 finishing (data not shown).

218 The large difference in "AGCT" counts could be due to the deletion of a genomic region
219 concentrating most of them, for instance within a repeated structure absent from the A-
220 clade isolates. However, Fig. 6 shows that this is not at all the case. In B- clade isolates,
221 the numerous occurrences of "AGCT" are rather uniformly distributed along the whole
222 genomes. However, we noticed that the "AGCT" distribution in the *P. neocaledonia*
223 genome exhibits a change of slope at one of its extremities, as if the corresponding
224 segment had been acquired from a A-clade strain. Such hypothesis was confirmed using a
225 dot-plot comparison with the *P. salinus* genome, to which this terminal segment is clearly
226 homologous (Fig. 7).

227

228 **“AGCT” was specifically deleted from A-clade pandoravirus genomes**

229 We have seen in the previous section that the extreme difference in the “AGCT”
230 count in *P. dulcis* (N=0) and *P. neocaledonia* (N=544) is not due to the local deletion of an
231 “AGCT”-rich segment. We then investigated if that difference was limited to “AGCT”, or if
232 other 4-mers exhibited large differences in counts. Fig. 8 shows that this was not the case.

233 If the frequencies of the various 4-mers within each genome exhibit tremendous
234 differences (very much at odd with their distribution in randomized sequences, see Fig.
235 4), the frequency for each 4-mer (low, average or high) was very similar across the two
236 different viral genomes (Spearman correlation, r=0.9859). The difference in “AGCT”
237 count is thus not the consequence of the use of globally distinct 4-mer vocabularies by
238 the two pandoravirus clades. It appears to be due to a selection specifically exerted
239 against the presence of “AGCT” in the genomes of A-clade pandoraviruses.

240 Another argument in favor of an active selection against the presence of “AGCT” is
241 provided by the following statistical computation. We first identified the orthologous
242 proteins in *P. dulcis* and *P. neocaledonia*, using the best-reciprocal Blastp match criterium.
243 We identified 585 orthologous ORFs. In *P. neocaledonia*, 180 of them were found to
244 contain one or several “AGCT” (for a total of 350 occurrences). We then computed the
245 average percentage of nucleotide identity in the alignments of these 180 *P. neocaledonia*
246 ORFs with their *P. dulcis* orthologous counterparts. The value was 69%.

247 According to a neutral scenario (and neglecting multiple hits), the probability is thus
248 $p = 0.69$ that any nucleotide remains the same along the evolutionary trajectory
249 separating the two pandoraviruses. For a given “AGCT”, the probability to remain intact

250 over the same evolutionary distance is $p_{intact} = 0.69^4 = 0.227$, such as none of the
251 four positions is changed. For the sake of simplicity, we will neglect the chance creation of
252 new "AGCT" during the process. As a result, we then expect *P. dulcis* orthologous ORFs to
253 exhibit 68 occurrences (i.e. 0.227×350) of "AGCT".

254 This simple calculation already indicates that the "AGCT" 4-mer diverged much faster (at
255 least 80 times faster since 350x0.227/80 < 1) than the rest of the orthologous coding
256 regions. This result suggests that the absence of "AGCT" in *P. dulcis* and *P. quercus*, as
257 well as its distinctive low frequency in all A-clade strains is the consequence of an active
258 counter selection. We discuss possible molecular mechanisms in the following section.
259 The above calculation could not be extended to interORFs regions, due to their much
260 lower conservation and their unreliable pairwise alignments.

261

262 ## Discussion

263 **Which model for the counter selection of "AGCT"?**

264 Following our statistical computations on random sequences confirmed by the
265 analysis of actual genome sequences, we can safely assume that the genome of the
266 common ancestor of the A- and B-clade pandoraviruses was not missing any 4-mers. Our
267 discussion will thus take for granted that the difference in "AGCT" frequency between the
268 two Pandoraviridae clades is the consequence of a loss in the A-clade rather than a gain in
269 the B-clade. Such phenomenon probably predated the split of the two clades as the
270 number of "AGCT" found in B-clade Pandoravirus genomes (≈500) is already 15 times
271 lower than expected in the corresponding randomized sequences (≈7800).

272

273 Any model proposed to explain our results must take into account that the two types of
274 pandoraviruses replicate with the same efficiency in various laboratory strains of
275 Acanthamoeba. From this we can reasonably assume that both clades do not differ much
276 in their range of natural hosts (one of which is known to be an Acanthamoeba for A-clade
277 *Pandoravirus inopinatum*[18]). The cause of the marked difference in "AGCT" counts
278 between the two clades must thus reside within the viruses themselves. Such inference is
279 further supported by the fact that none of the other families of giant viruses[19] infecting
280 the very same Acanthamoeba hosts exhibit a similar 4-mer anomaly in their genome
281 composition.

282 The first model that comes to mind is inspired from the well-documented restriction-
283 modification systems that many bacteria use to counteract bacteriophage infections. The
284 host bacterial cells express DNA sites (most often short palindromes) specific
285 endonucleases that cut the invading phage genome before it could replicate. Such
286 defense mechanism imposes the bacteria to protect the cognate motif in its own genome
287 using a specific methylase. According to the Red Queen evolutionary concept, the
288 bacteriophages could counteract the host's defense by removing the targeted site from
289 their own genome[17]. The absence of the palindrome "GCGC" that we previously noticed
290 in several large enterobacterial phages[13-16] could result from such evolutionary strategy.

291 Translating such a model in our system thus requires three distinct assumptions: 1) that
292 the Acanthamoeba cells express an antiviral endonuclease specific for "AGCT"; 2) that B-
293 clade pandoraviruses are immune from it (as other Acanthamoeba-infecting viruses); 3)

294 that A-clade pandoraviruses evolved a different strategy by removing the endonuclease
295 target from their genomes.

296 Such a model was readily invalidated by simply attempting to digest the B-clade *P.*
297 *neocaledonia* genomic DNA (extracted from infectious particles) with commercial
298 restriction enzymes (such as PvuII) targeting "cAGCTg" (212 occurrences) and AluI,
299 targeting "AGCT" (544 occurrences). The resulting Pulsed-field gel electrophoresis (PFGE)
300 pattern showed that these sites were not protected (Fig. 9). Accordingly, the PacBio data
301 used to sequence the *P. neocaledonia* genome[2] did not indicate the presence of modified
302 nucleotides at the "AGCT" sites[20].

303 We must point out that the above results simultaneously invalidate a symmetrical model
304 where the "AGCT"-specific endonuclease would have been encoded by the
305 pandoraviruses, together with the protective cognate methylase. Such a hijacked
306 restriction/modification system would have been attractive as it is found in
307 chloroviruses[21], another family of large eukaryotic DNA viruses. Unfortunately, it does not
308 apply here. Accordingly, no homolog of the cognate DNA-methyl transferase was
309 detected among the *P. neocaledonia* or *P. macleodensis* protein-coding gene contents.
310 Further nailing the coffin of such restriction/modification hypothetical model, no
311 difference in terms of potentially relevant endonuclease or DNA methylase was found
312 between the gene contents of the A-clade *P. dulcis* and *P. quercus* and those of the B-
313 clade *P. neocaledonia* and *P. macleodensis*.

314 A more hypothetical model would assume that the "AGCT" motif is targeted at the
315 transcript level (i.e. "AGCU") rather than at the DNA level. Classical endonucleases and

316 DNA methylases would thus not be involved in the host-virus confrontation. There are
317 several arguments against a mechanism directly targeting viral transcripts.

318 First, as B-clade pandoraviruses exhibit similar proportions of "AGCT" in ORFs and inter-
319 ORF regions, the A-clade strains would have had no incentive to eliminate the motif from
320 their intergenic regions, as *P. dulcis* and *P. quercus* have done totally in reaching zero
321 occurrences. "AGCT" is also still present in some protein-coding regions of *P. inopinatum*
322 (N=15), *P. salinus* (N=3), and *P. celtis* (N=1).

323 Second, very few motif-specific RNAses are known, and to our knowledge, only one is
324 viral: a protein encoded in the bacteriophage T4 RegB gene[22]. We found no significant
325 homolog of this protein in the pandoraviruses or Acanthamoeba. We also looked for
326 mRNA methylases that could act as a protective mechanism for the viral transcript. A
327 single one was described in another family of eukaryotic DNA virus: the product of the
328 Megavirus Mg18 gene[23]. Again, no significant homolog of this protein was detected in the
329 pandoraviruses.

330 In conclusion to this section, if the presence of "AGCT" decreases the virus fitness, we
331 found no evidence that it is due to a DNA or RNA nuclease-mediated defense mechanism
332 in Acanthamoeba. However, it could still be due to an unknown inhibitory mechanism
333 acting at the transcription regulation level to which B-clade pandoviruses would exhibit
334 some immunity. The corresponding proteins could be encoded among the numerous
335 ORFans found in pandoravirus genomes[1-3]. Alternatively, the "AGCT" deficit could be due
336 to a restriction imposed by unknown additional hosts in nature, although quite an unlikely
337 scenario given the ubiquity and abundance of Acanthamoeba in the environment.

338 Finally, could “AGCT” be deleterious for some intrinsic reasons, for instance due to its
339 palindromic structure and composition? This is very unlikely, when one compare the
340 absent “AGCT” in *P. dulcis* and *P. quercus*, with other 4-mers with identical structures and
341 compositions. For instance “ACGT” occurs at 5822 and 6165 positions (in P. dulcis and P
342 quercus, respectively), and “GATC” occurs at 8114 and 8567 times) in (P. dulcis and P.
343 quercus, respectively). The presence or absence of “AGCT” does not either exert a strong
344 constraint on protein sequences, as the amino-acids encoded by “AGC” or “GTC” (Serine
345 and Alanine, respectively) have many possible alternative codons and are easily
346 replaceable residues given their mild physicochemical properties. Finally, we found no
347 evidence that the removal of “AGCT” was due to a specific (for instance, enzyme-
348 mediated) process targeting then replacing the forbidden 4-mer by a constant alternative
349 word. Replacement patterns for 72 *P. dulcis* sites unambiguously mapped to their
350 homologous *P. neocaledonia* “AGCT” counterparts are indicated in Table 2. It suggests
351 that the complete loss of “AGCT” in the A-clade strains is due to a stringent, nevertheless
352 random (i.e. non-directed) evolutionary process.

353 The analysis of long nucleotide (and amino acid) sequences as overlapping k-mers
354 has a long history in bioinformatics. Initially proposed in the context of the RNA folding
355 problem[23], the concept was then quickly applied to many other areas including gene
356 parsing[24], the detection of regulatory motifs[25, 26], and has become central to the fast
357 implementation of large-scale similarity search[27, 28], sequence assembly[29], and the binning
358 of metagenomics data[30, 31]. However, its popularity should not hide that most of the
359 observed frequency disparities (starting from the simplest mononucleotide composition)
360 between k-mers within a given organism, or across species have not yet received
361 convincing biological explanations[32, 33]. This suggests that profound and unexpected

362 biological insights may one day come out from the analysis of k-mer frequencies, and in
363 particular from their most improbable fluctuations. In a daring parallel with the delayed
364 understanding of the CRISPR/CAS system from the initial spotting of intriguing repeats[34],
365 we would like to expect that the pandoraviridae "AGCT" distribution anomaly might lead
366 to the discovery of a novel defense mechanism against viral infection.

367

368 ## Materials and Methods

369 **Chaos game representation**

370 Chaos game representation (CGR) was introduced in 1990 by Jeffrey[6] to visually detect
371 global patterns in large DNA sequences. It was inspired from a method generating fractals
372 within a polygon as a sequence of points, iteratively positioned according to a rule based
373 on their distance to one of the vertices of the polygon. To apply this method to DNA
374 sequences, one uses a square with corners labelled A, T, G and C. Starting from the
375 center of the square, the sequence is used to determine the position of the next point at
376 the center of the line connecting the previous point and the corner corresponding to the
377 current nucleotide. In addition to global patterns, the resulting graph also reveals the
378 differential frequencies of substrings (k-mers), for instance leaving a blank area at the
379 position corresponding to a missing substring (Fig. 2). CGR thus allows the rapid detection
380 of compositional anomaly of k-mers for increasing n values, instead of comparing large
381 statistical tables. Once the k-mer (4-mer) distributions of interest were determined by
382 CGR, they were further analyzed and compared using a standard counting package[8].

383

384 **Pulse-field gel electrophoresis (PFGE)**

385 Approximately 5,000 pandoravirus particules were embedded in 1% low gelling agarose
386 and the plugs were incubated in lysis buffer (50mM Tris-HCl pH8.0, 50mM EDTA, 1% (v/v)
387 N-laurylsarcosine, 1mM DTT and 1mg/mL proteinase K) for 16h at 50°C. After lysis, the
388 plugs were washed once in sterile water and twice in TE buffer (10mM Tris-HCl pH8.0 and
389 1mM EDTA) with 1mM PMSF, for15 min at 50°C. The plugs were then equilibrated in the
390 appropriate restriction buffer and digested with 20 units of PvuII or AluI at 37°C for 14
391 hours. Digested plugs were washed once in sterile water for 15 min, once in lysis buffer
392 for 2h and three times in TE buffer. Electrophoresis was carried out in 0.5X TAE for 18 h at
393 6V/cm, 120° included angle and 14°C constant temperature in a CHEF-MAPPER system
394 (Bio-Rad) with pulsed times ramped from 0.2s to 120s.

395

396 **Availability of data**

397 All virus genome sequences analyzed in this work are freely available from the public
398 GenBank repository (URL://www.ncbi.nlm.nih.gov/genbank/). The Pandoravirus
399 sequences used here correspond to the following accession numbers: *P. dulcis*
400 (NC_021858), *P. neocaledonia* (NC_037666), *P. macleodensis* (NC_037665), *P. salinus*
401 (NC_022098), *P. quercus* (NC_037667), *P. celtis* (NC_), *P. inopinatum* (NC_026440), *P.*
402 *pampulha* (LT972219.1), *P. massiliensis* (LT972215.1), *P. braziliensis* (LT972217).

403

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493

494 **Figure Legends**

495 **Figure 1. Phylogenetic structure of the Pandoraviridae.** Adapted from [ref. 3]. The
496 number of occurrences of the "AGCT" 4-mer is indicated for the genome of each strain.
497 The counts are given for one DNA strand and are identical for both strands ("AGCT" is
498 palindromic).
499
500 **Figure 2. Chaos game representation of the *P. dulcis* genome.** The largest square left
501 blank (circled in red) corresponds to "AGCT", indicating the absence of this 4-mer in the
502 genome.
503
504 **Figure 3. Influence of random sequence length on the number of missing 4-mers.** 10.000
505 random sequences up to 10.000 bp in size were analyzed. Except for extremely rare
506 fluctuations, no sequence longer than 4000 bp exhibits a missing 4-mer. 4-mer overlaps
507 as well as nucleotide compositions are taken into account in this analysis.
508
509 **Figure 4. Distribution of 4-mer frequencies in natural and randomized genome**
510 **sequences.** Top: histogram of the number of distinct 4-mers occurring at various numbers
511 of occurrences in the *P. dulcis* genome; Bottom: same analysis after randomization.
512
513 **Figure 5. Missing 4-mers in the largest viral genomes.** Except for *P. dulcis* and *P. quercus*,
514 the largest viral genomes missing a 4-mers are those of 5 distinct bacteriophages
515 (accession numbers: NC_019401, NC_025447, NC_027364, NC_027399, NC_019526).
516

517 **Figure 6. Cumulative distribution of "AGCT" occurrences along the different**

518 **pandoravirus genomes.** The "AGCT" word appears uniformly spread throughout the B-

519 clade pandoravirus genomes, except for a clear rarefaction at the end of the P.

520 neocaledonia genome sequence.

521

522 **Figure 7. DNA sequence dot-plot comparison of *P. neocaledonia* (horizontal) and *P.***

523 ***salinus* (vertical).** The two genomes only exhibit remnants of collinearity except for the

524 terminal region of *P. neocaledonia* (red circle) coinciding with a low "AGCT" density

525 typical of A-clade strains (Fig. 6). Dot plot generated using GEPARD[35] with parameters:

526 word size=15, window size=0.

527

528 **Figure 8. Comparison of the proportion of all 4-mers in *P. dulcis* (A-clade) vs. *P.***

529 ***neocaledonia* (B-clade).** The 4 most frequent 4-mers are "GCGC", "CGCG", "CGCC", and

530 "GGCG".

531

532 **Figure 9. Digestion of *P. neocaledonia* DNA at "AGCT" sites.** Lane 1: undigested *P.*

533 *neocaledonia* DNA (2.2 Mb) migrating as expected. The bottom band (below 48.5 kb)

534 correspond to an episome not always present. Lane 2: *P. neocaledonia* DNA digested by

535 the PvuII restriction enzyme (cutting site: cAGCTg). Lane 3: *P. neocaledonia* DNA digested

536 by the AluI restriction enzyme (cutting site: AGCT). These results demonstrate that the

537 "AGCT" sites are not protected by modified nucleotides.

538

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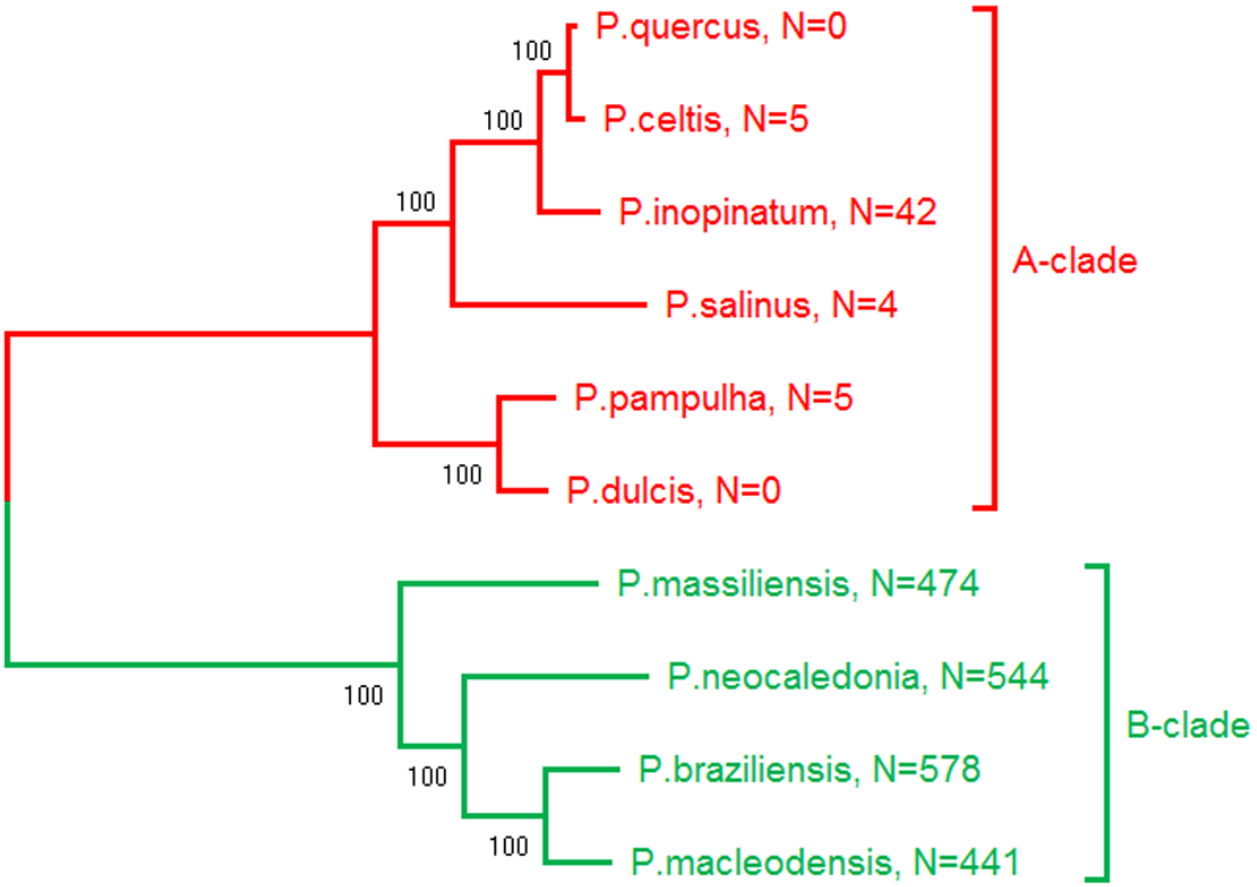
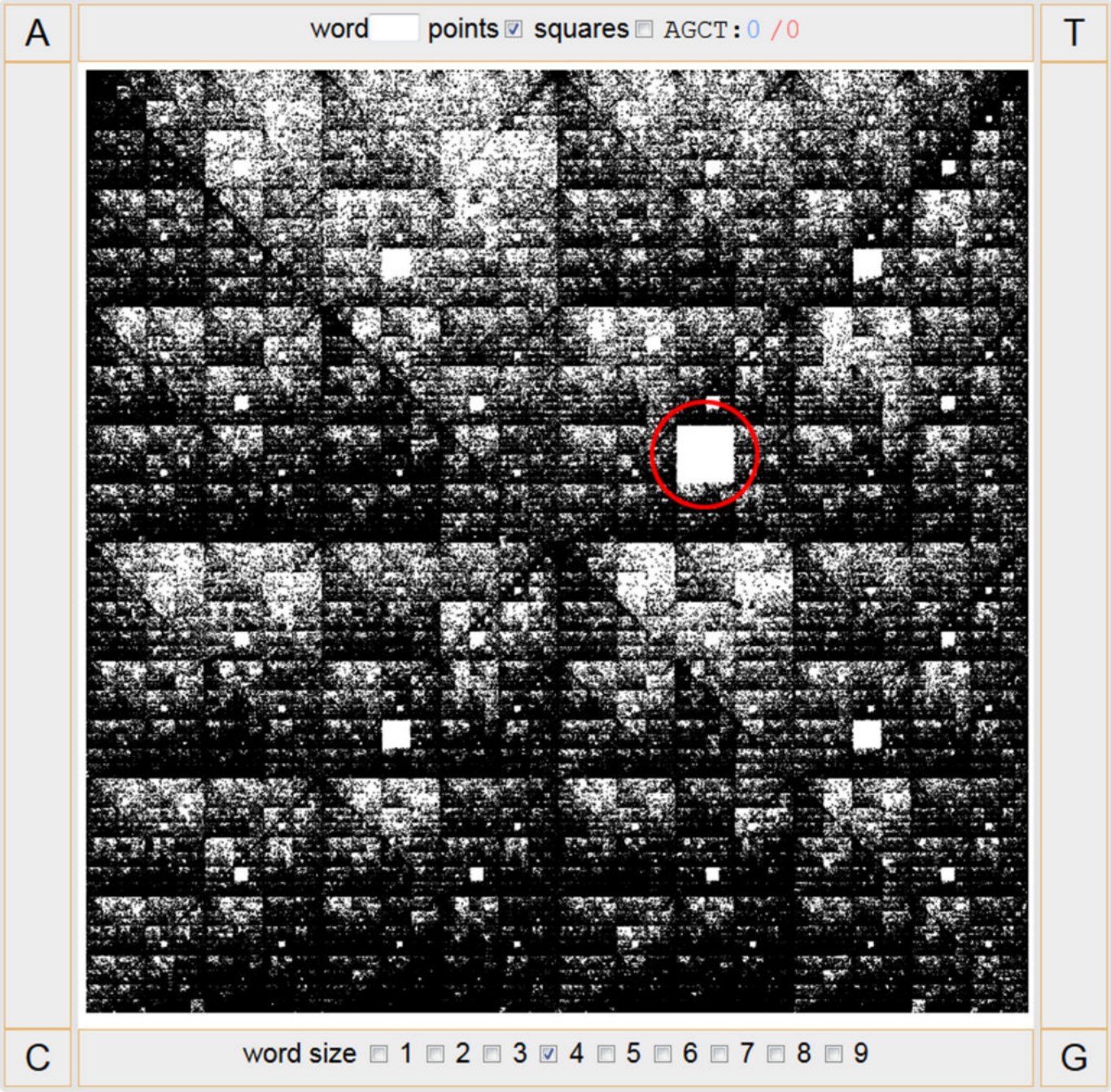
549

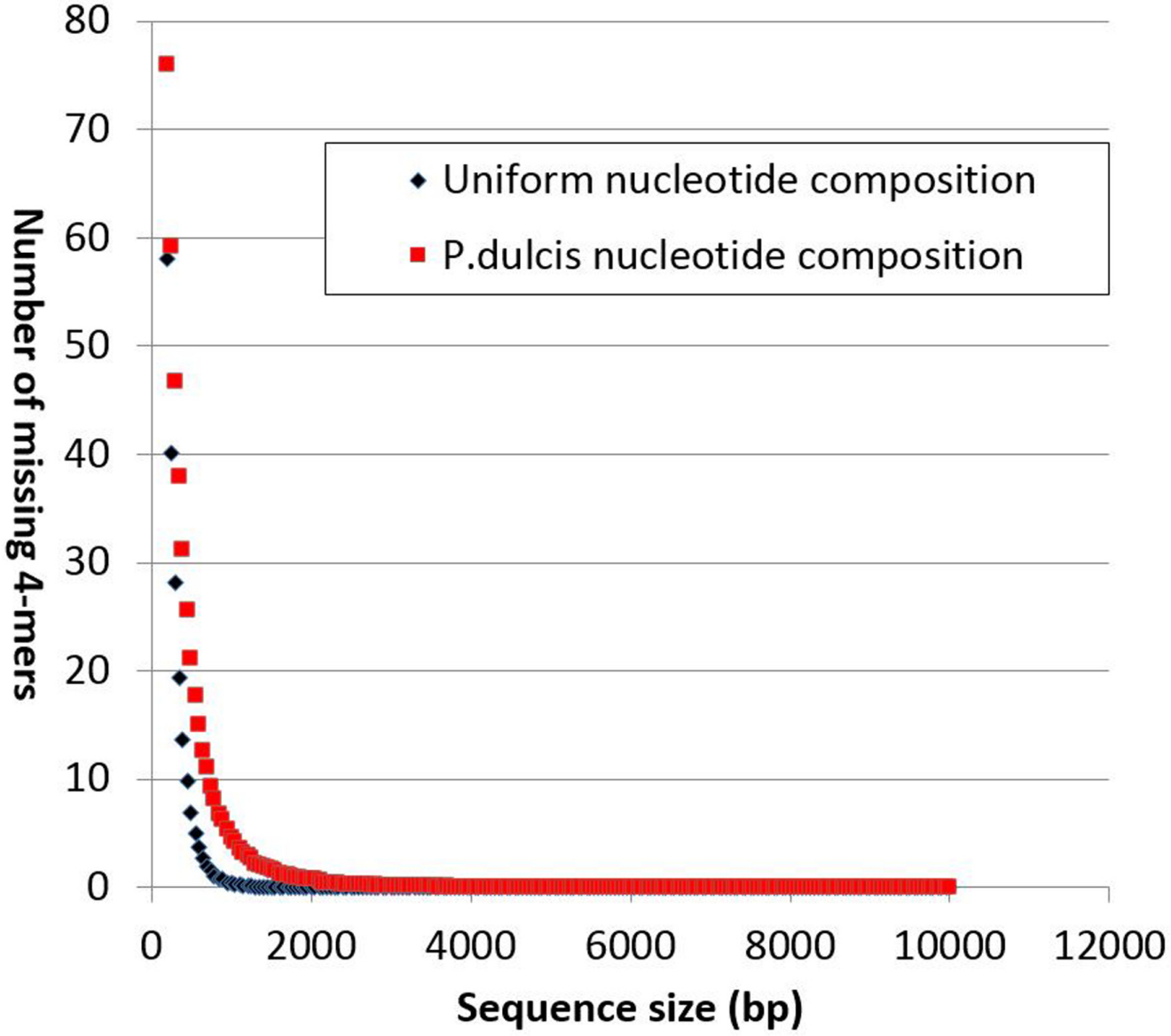
550 ## Competing interests

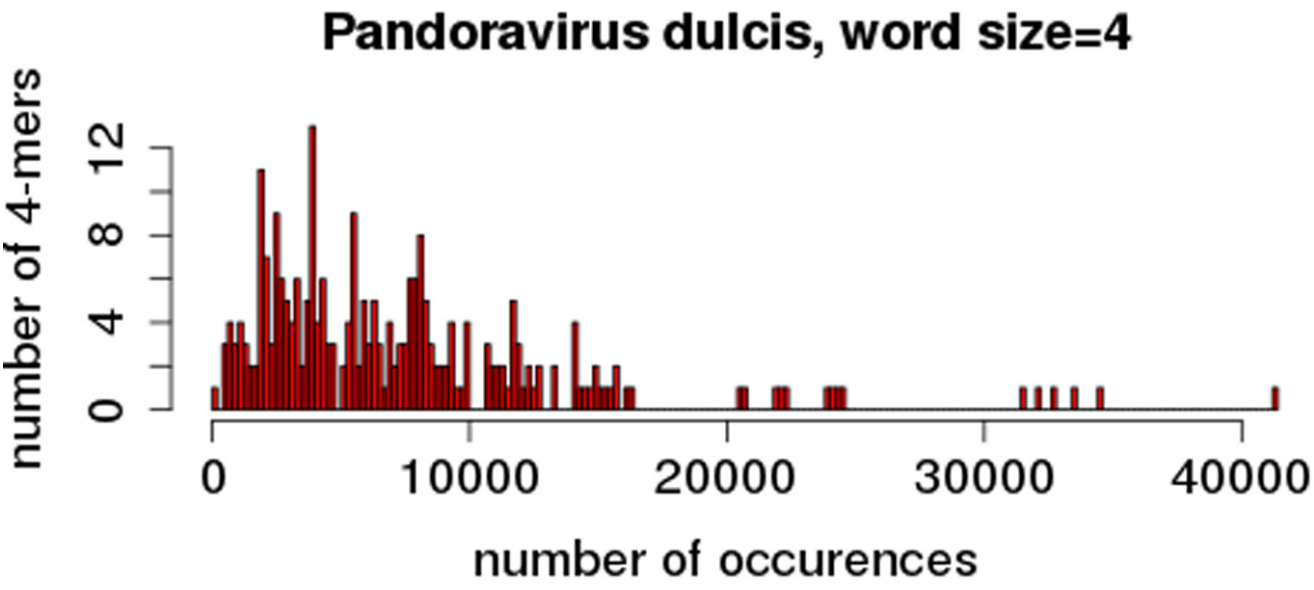
551 The authors declare that they have no competing interests

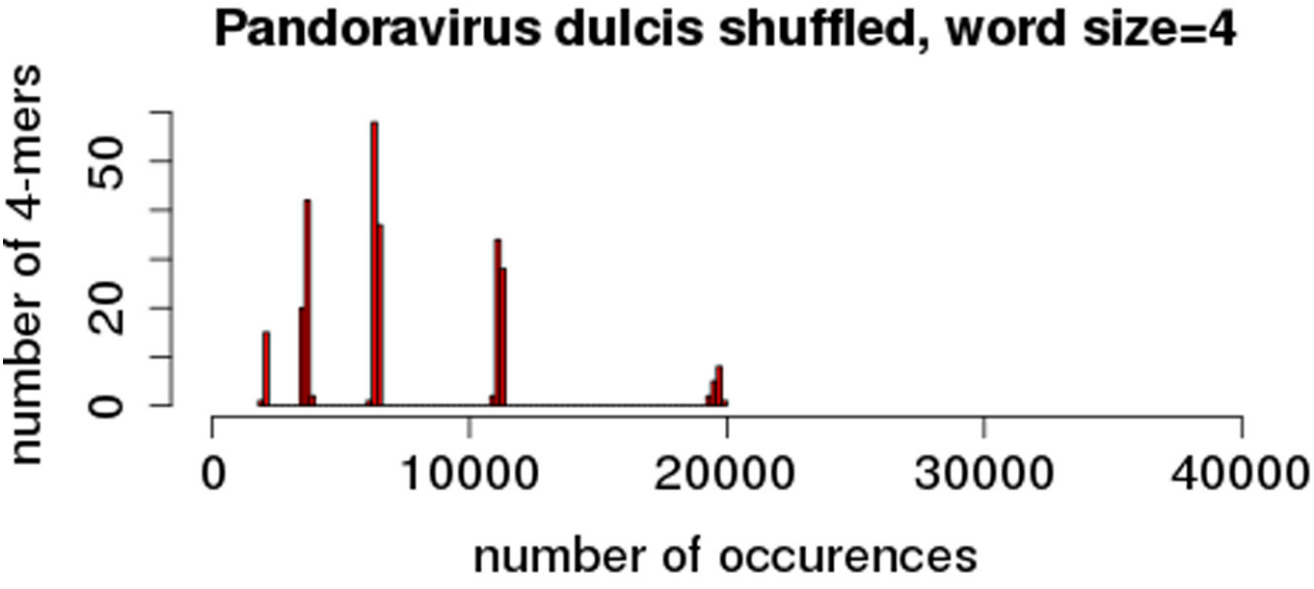
552

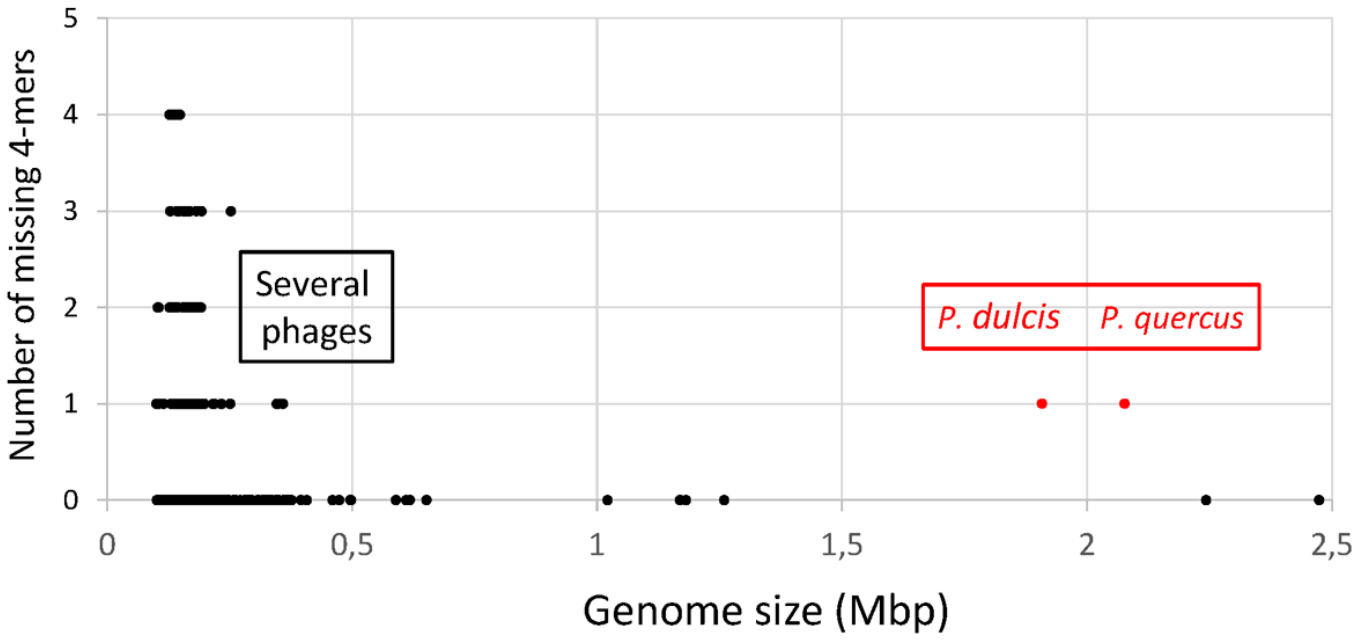

P.quercus, N=0
P.celtis, N=5
P.inopinatum, N=42
P.salinus, N=4
P.pampulha, N=5
P.dulcis, N=0
A-clade
P.massiliensis, N=474
P.neocaledonia, N=544
P.braziliensis, N=578
P.macleodensis, N=441
B-clade
100
100
100
100
100
100
100

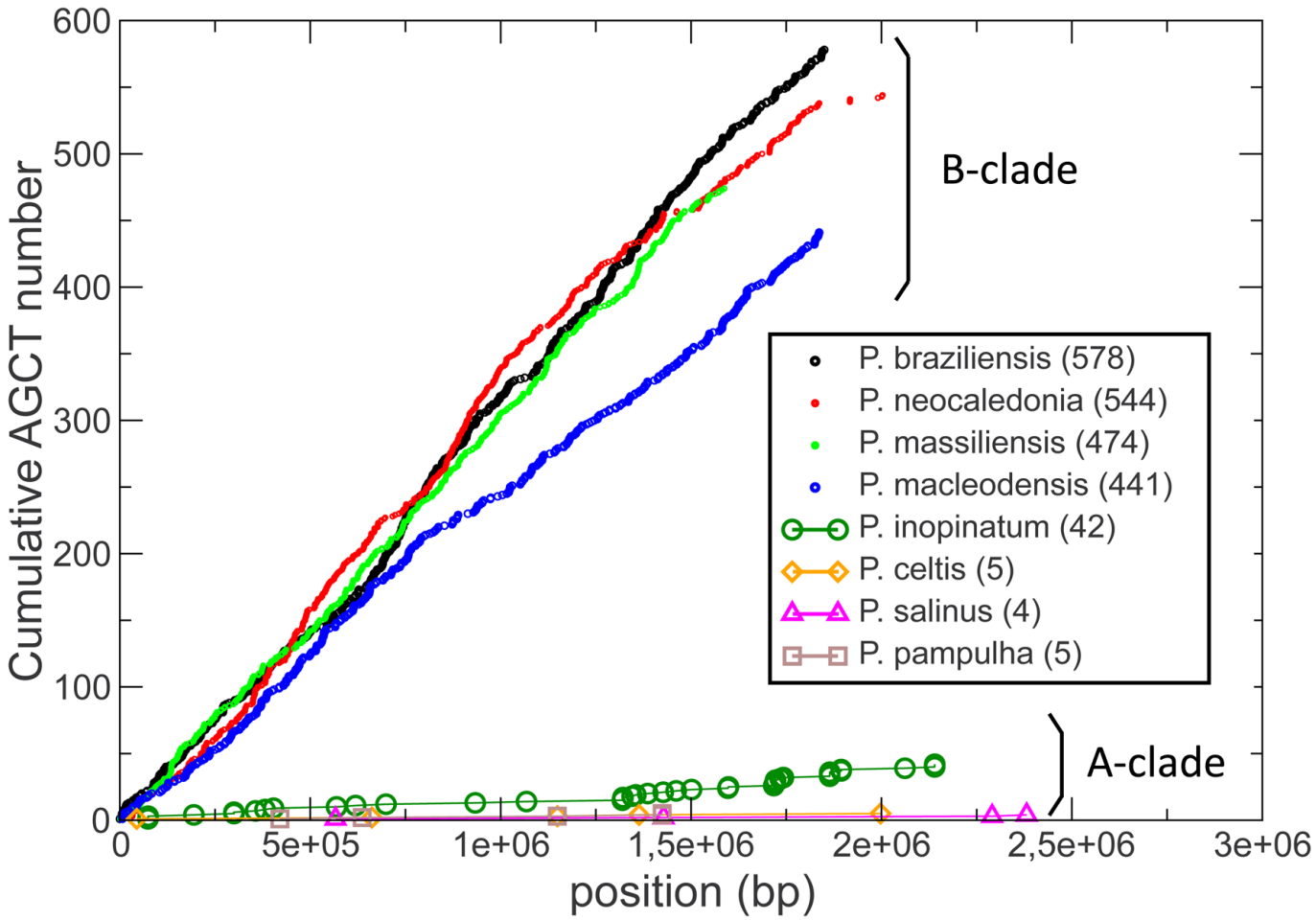

A
word
points
squares
AGCT: 0 / 0
T
C
word size 1 2 3 4 5 6 7 8 9
G

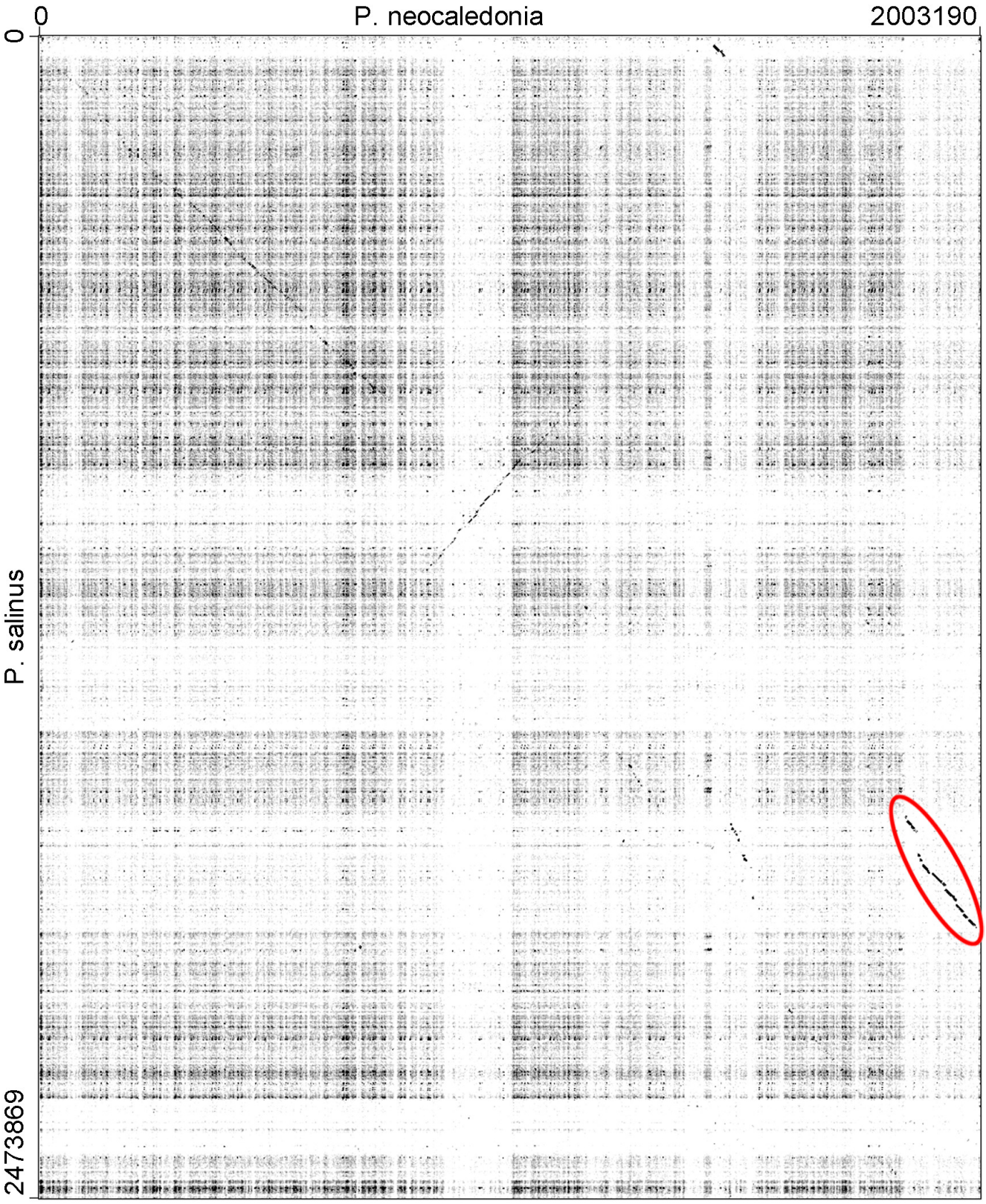

80
70
60
50
40
30
20
10
0
Number of missing 4-mers
Uniform nucleotide composition
P.dulcis nucleotide composition
0
2000
4000
6000
8000
10000
12000
Sequence size (bp)

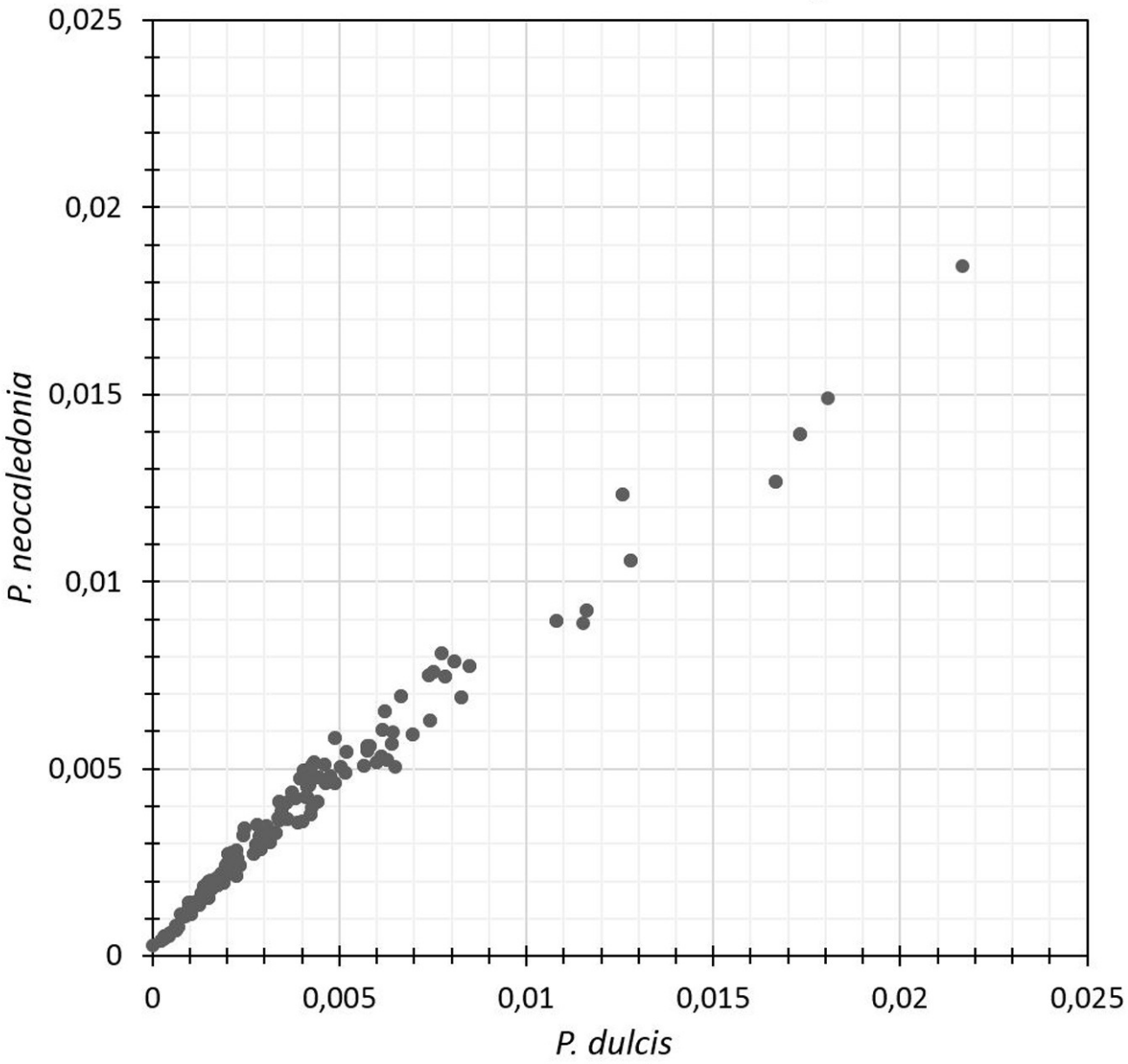

Pandoravirus dulcis, word size=4
number of 4-mers
0
4
8
12
0
10000
20000
30000
40000
number of occurences


Pandoravirus dulcis shuffled, word size=4
number of 4-mers
0
20
50
0
10000
20000
30000
40000
number of occurences


Number of missing 4-mers
5
4
3
2
1
0
Several phages
P. dulcis
P. quercus
0
0,5
1
1,5
2
2,5
Genome size (Mbp)


600
500
400
300
200
100
0
Cumulative AGCT number
0
5e+05
1e+06
1,5e+06
2e+06
2,5e+06
3e+06
position (bp)
B-clade
A-clade
P. braziliensis (578)
P. neocaledonia (544)
P. massiliensis (474)
P. macleodensis (441)
P. inopinatum (42)
P. celtis (5)
P. salinus (4)
P. pampulha (5)


0
P. neocaledonia
2003190
0
P. salinus
2473869


0,025
0,02
0,015
0,01
0,005
0
P. neocaledonia
0
0,005
0,01
0,015
0,02
0,025
P. dulcis

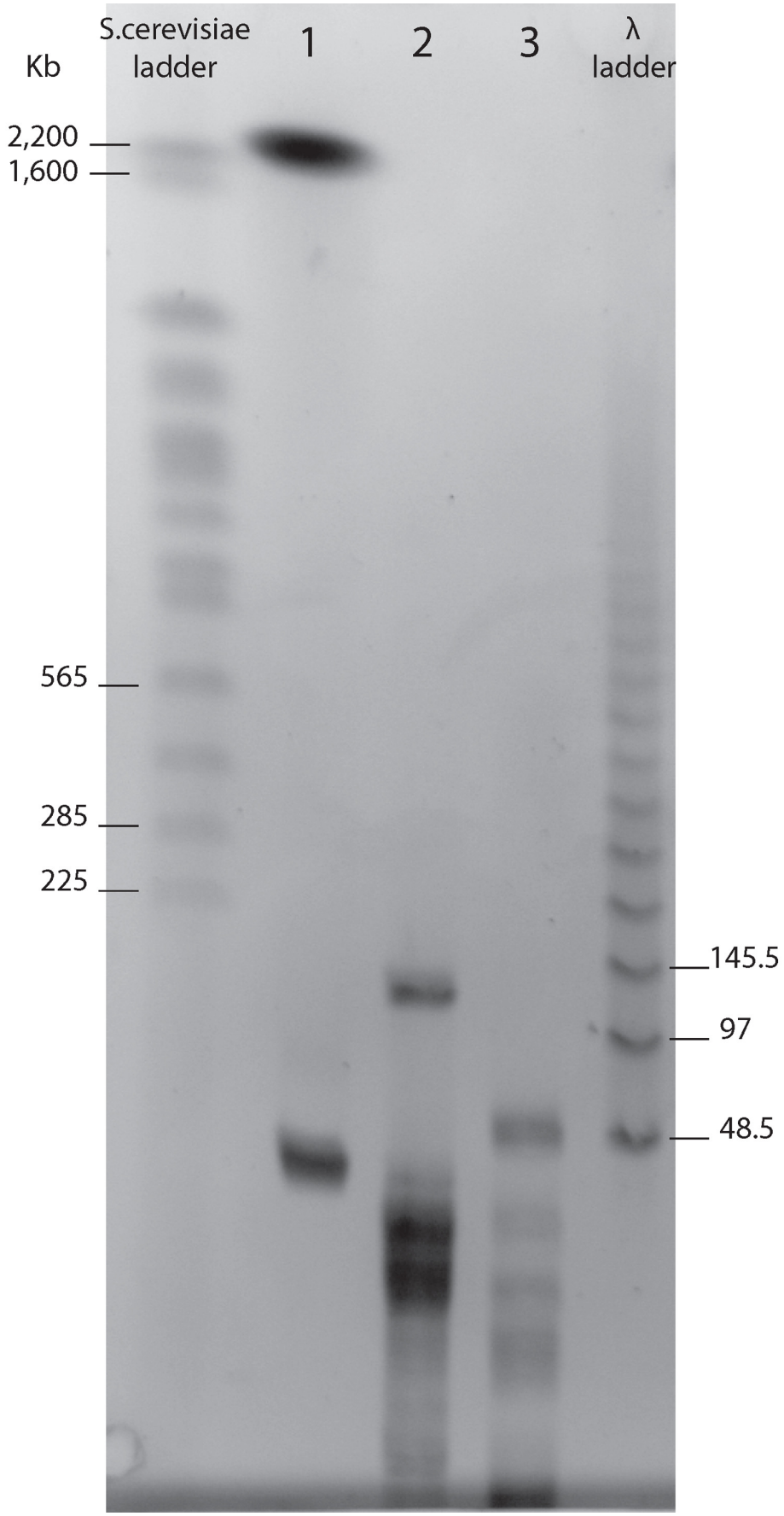

Kb
S.cerevisiae ladder
1
2
3
λ ladder
2,200
1,600
565
285
225
145.5
97
48.5

Table 1. Distribution of the AGC (and the complementary GCT) 3-mers

Statistics	P. dulcis			P. quercus		
Genome size (bp)	1,908,524			2,077,288		
	interORF	ORF	global	interORF	ORF	global
AGC frequency (strand 1)	0.0101 (1/99)	0.0112 (1/89)	0.0109 (1/92)	0.0098 (1/102)	0.0110 (1/90)	0.0106 (1/94)
GCT frequency (strand 1)	0.0102 (1/98)	0.0156 (1/64)	0.0138 (1/72)	0.0097 (1/103)	0.0145 (1/68)	0.0129 (1/77)
AGC/GCT (2 strands, global)	0.0123 (1/81)			0.0118 (1/85)		
AGC/GCT overall rank	37/64			43/64		
p(AGC).p(T)	$2.24\ 10^{-3}$ (1/446)			$2.31\ 10^{-3}$ (1/432)		
AGCT expected number (one strand x p(AGC).p(T))	4286			4898		
AGCT observed number	0			0		
ACGT expected number (one strand x p(ACG).p(T))	7884			8387		
ACGT observed number	5822			6165		

Table 2. *Homologous site replacements between P. neocaledonia and P. dulcis.*

***P. neocaledonia* → *P. dulcis* variant**	**Number**
AGCT → AGTT	**31**
AGCT → AACT	**18**
AGCT → GGCT	**4**
AGCT →AACC	**4**
AGCT →AATT	**3**
AGCT →GGCG	**2**
AGCT→[ACGA,ACTT,AGAT,AGCC,AGGC, CATT, GGCC, GGTT, GTCT, TGCC, TGGT, TGTC]	**1**