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18 **Rapid recovery of nuclear and mitochondrial genes by genome skimming from**
19 **Northern Hemisphere freshwater crayfish**
20
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24 **# Equal contribution**
25
26 **Northern crayfish evolution**
27 FREDERIC GRANDJEAN *et al.*
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77 Grandjean, F. (2016) Rapid recovery of nuclear and mitochondrial genes by genome
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79 000-000.

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81 Molecular phylogenetics has benefited tremendously from the advent of next-
82 generation sequencing, enabling quick and cost-effective recovery of whole
83 mitogenomes via an approach referred to as "genome-skimming". Recently, genome
84 skimming has been utilised to recover highly-repetitive nuclear genes such as 18S and
85 28S ribosomal RNA genes that are useful for inferring deeper evolutionary
86 relationships. To address some outstanding issues in the relationships among Northern
87 Hemisphere freshwater crayfish (Astacoidea), we sequenced the partial genome of
88 crayfish species from Asian, North American and European genera and report the
89 successful recovery of whole mitogenome sequences in addition to three highly-
90 repetitive nuclear genes namely, histone H3, 18S and 28S ribosomal RNA. Consistent
91 with some previous studies using short mtDNA and nuclear gene fragments,
92 phylogenetic analyses based on the concatenation of recovered mitochondrial and/or
93 nuclear sequences recovered the Asian cambarid lineage as basal to all astacids and
94 North American cambarids, which conflicts with the current taxonomic classification
95 based on morphological and reproduction-related characters. Lastly, we show that
96 complete H3, 18S and 28S ribosomal RNA genes can also be consistently recovered

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97 from a diverse range of animal taxa, demonstrating the potential wide utility of
98 genome skimming for nuclear markers.
99
100

101 **Keywords**: Genome skimming, mitogenomes, nuclear markers, Astacidae,
102 Parastacidae, Cambaridae
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111 Introduction

112

113 Estimation of evolutionary relationships among organisms using DNA
114 sequence information is now an established part of comparative biology due to the
115 PCR and automated Sanger sequencing revolution since the late 1990s (Avise et al.
116 2000; Hillis et al. 1996). Early in this phylogenetic revolution, most studies utilised
117 sequences of single mitochondrial genes such as the 16S ribosomal RNA, cytochrome
118 b, cytochrome oxidase I or nuclear genes especially the 18S ribosomal RNA. Recent
119 studies have increasingly used nucleotide data from multiple mitochondrial genes and
120 several nuclear genes, resulting in larger datasets commonly in the order of 5,000 to
121 10,000 bp. The development of next-generation sequencing (NGS) technology has led
122 to rapidly declining costs for DNA sequencing and shows promise in producing
123 datasets comprising of hundreds, if not thousands of loci or characters.
124 Assembling datasets using PCR-based methods presents several challenges
125 especially with the increasing expectations that multiple loci are required for robust
126 phylogenies. This amounts to thousands of base pairs and is often coupled with the
127 need for adequate taxon samples, requiring up to and even in excess of 100 samples.
128 These challenges become amplified with the use of museum-held specimens, which
129 have many advantages in relation to supporting biodiversity-related and phylogenetic
130 studies (Graham et al. 2004; McCormack et al. 2015; Suarez & Tsutsui 2004; Thomas

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131 et al. 1990). Tissue samples from museum specimens are often limited in volume and
132 are usually characterised by highly-degraded DNA requiring multiple rounds of short
133 amplicon sequencing often with low success rate (Andersen & Mills 2012; Aznar-
134 Cormano et al. 2015; Li et al. 2015; McCormack et al. 2015; Tin et al. 2014). The use
135 of next-generation sequencing rather than Sanger sequencing can reduce the cost of
136 this approach, often referred to as Targeted Amplicon Sequencing (Bybee et al. 2011).
137 Nevertheless, this does not overcome the time and cost of multiple and failed PCR
138 reactions which will be common for degraded samples, and also the bioinformatics
139 workload of assembling and aligning data from multiple short fragments (Meimberg
140 et al. 2016).

141 With high throughput sequencing, it is now possible to sequence the genome
142 of eukaryotic organisms for a few thousand dollars in a matter of weeks (Goodwin et
143 al. 2016). However, it is still costly and time-consuming to generate sufficient
144 sequences for robust phylogenetic reconstruction, while also maximising taxon
145 sampling. Currently, the two most popular methods for generating sizable
146 phylogenomic datasets are: (1) the anchored hybrid enrichment approach (Lemmon et
147 al. 2012; Ruane et al. 2015) and (2) the ultra-conserved element procedure (Faircloth
148 et al. 2012; McCormack et al. 2015). Other methods are being developed to exploit
149 museum samples with highly-degraded DNA but require whole genome resources for
150 read mapping (Tin et al. 2014).

151 Alternatively, a simple, rapid, and low-cost method of rapidly assembling
152 datasets of approximately 10-15 kbp is to use an NGS-based approach involving
153 partial genome scans of samples, also referred to as genome skimming (Gan et al.
154 2014; Malé et al. 2014; Straub et al. 2012; Tan et al. 2015). Most animal genome
155 skimming studies have focused on mitochondrial sequences that are present in many
156 copies in the eukaryotic cell. Mitochondrial genomes have reduced intergenic
157 elements making them straightforward to recover, assemble, and annotate using a
158 suite of bioinformatics methods and pipelines (Bernt et al. 2013; Hahn et al. 2013;
159 Malé et al. 2014), some of which also facilitate phylogenetic analysis (Tamura et al.
160 2013; Tan et al. 2015). A major drawback of this approach is that phylogenies based
161 on mitochondrial sequences may not be reflective of the full evolutionary history of
162 the organisms under study as represented by their nuclear genomes (Timm &
163 Bracken-Grissom 2015).

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164 In this regard, an important recent development is the discovery that repetitive
165 nuclear genetic elements, predominately from the nuclear ribosomal cluster, can also
166 be recovered by genome skimming. The data from these partial genome scans, often
167 representing less than 1% of the genome, contain sufficient reads from repetitive
168 nuclear genes to allow them to be routinely recovered for phylogenetic studies
169 (Besnard et al. 2016; Dodsworth et al. 2015; Kocher et al. 2015; Kocher et al. 2014;
170 Malé et al. 2014; Richter et al. 2015; Straub et al. 2012). In this study, we demonstrate
171 the wide utility of genome skimming to 22 species of Northern and Southern
172 Hemisphere freshwater crayfish. We show that, in addition to extracting the full
173 mitogenomes for each species, it is also possible to recover the complete 18S rRNA,
174 28S rRNA and histone (H3) nuclear gene sequences from a fraction of a MiSeq run
175 (approximately 800 Mbp output). All three of these nuclear genes are considered
176 especially useful for establishing deeper level relationships as demonstrated by a
177 number of studies on crustaceans, including freshwater crayfish (Bracken-Grissom et
178 al. 2014; Bybee et al. 2011; Toon et al. 2010), that utilised information from these
179 genes using PCR-based methods.
180 While freshwater crayfish have been subject to a number of molecular genetic
181 studies that use conventional PCR-based approaches, there are still outstanding issues
182 concerning phylogenetic relationships among major groups within each superfamily
183 (Braband et al. 2006; Bracken-Grissom et al. 2014; Toon et al. 2010). One of the
184 persistent issues in freshwater crayfish systematics is the unresolved phylogenetic
185 placement of the Asian freshwater genus *Cambaroides*. Though this genus is
186 taxonomically placed in the family Cambaridae, it is often recovered as sister to
187 species of Astacidae or in a basal position rather as sister to other members of
188 Cambaridae from North America. While several studies have used molecular data to
189 study relationships among Northern Hemisphere crayfish species, these often have
190 limitations with respect to taxon sampling (either limited or unbalanced) and number
191 of molecular characters (Ahn et al. 2006; Braband et al. 2006; Bracken-Grissom et al.
192 2014), sometimes producing conflicting results. In this study, we assemble the
193 complete mitochondrial genomes from ten species of European, North American and
194 Asian crayfish and one lobster species. We also use genome skimming to recover
195 complete or near-complete sequences of nuclear 18S and 28S RNA genes and the
196 histone H3 gene from these samples plus data from 11 additional species of crayfish
197 and lobsters. Our phylogenetic analyses show that the mitochondrial and nuclear trees

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198 are fully congruent and the combined dataset produces trees with consistently high
199 nodal support that indicates the polyphyly of the Cambaridae. We also demonstrate
200 that our genome skimming approach recovers the same three nuclear genes from
201 samples of a number of major animal groups (e.g. Mammalia, Teleostei, Aves,
202 Mollusca, Arthropoda), suggesting this approach has wide utility for animal
203 molecular systematics.
204
205 **Material and Methods**
206
207 *Sampling and sequencing*
208
209 For this study, ten Northern Hemisphere freshwater crayfish samples
210 belonging to the superfamily Astacoidea and one lobster sample in the superfamily
211 Nephropoidea that do not have mitogenome representative sequences on NCBI were
212 acquired from various geographical locations (marked with '*' in Supplementary
213 Data 1), identified based on morphology and further validated with nucleotide
214 similarity searches against publicly available COI, 16S and 12S rRNA gene fragments
215 for the corresponding species (Supplementary Data 2). For *Cambaroides similis*,
216 whose mitogenome is already available on NCBI, a new additional sample of the
217 same species was collected from Korea (94% identity from a 810 bp alignment to the
218 *C. similis cox1* gene from NC_016925.1) to further scan for nuclear genes. For the
219 *Astacus* species, due to low mitogenome content in the muscle tissue, additional
220 isolates of each species were further enriched for mitochondria (Grandjean et al.
221 1997) and sequenced to recover the complete mitogenome. For all samples,
222 purification of ethanol-preserved tissues and partial whole genome sequencing on the
223 Illumina MiSeq (2 × 250 bp or 2 × 150 bp) was carried out as described in Gan et al.
224 (2014) at the Monash University Malaysia Genomics Facility.
225 Several species from other superfamilies, Parastacoidea (Southern Hemisphere
226 crayfish) and Nephropoidea (lobsters), were also included in this study for
227 comparative purposes or as outgroup species to the Northern Hemisphere crayfish
228 group. For these taxa, nuclear gene sequences or raw sequence read datasets were
229 recovered from various sources – existing mitogenome and nuclear sequences for
230 some species were obtained from NCBI (accession numbers cited in Supplementary

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231 Data 1), whereas raw sequence reads for other species were available from previous
232 mitogenome studies by our group (studies also cited in Supplementary Data 1).
233
234 *Genome skimming for mitochondrial and nuclear sequences*
235
236 Genome skimming was performed according to the workflow illustrated in
237 Fig. 1. Sequences generated from the partial genome sequencing of each sample were
238 initially pre-processed using Trimmomatic (Bolger et al. 2014) to remove adapters
239 and low quality sequences (*Illumina clip 2:30:10, sliding window 4:20, leading: 3,*
240 *trailing 3, min length 100*). The resulting quality-filtered reads were then assembled
241 using: 1) IDBA-UD (Peng et al. 2012), an iterative *de novo* assembler for data with
242 uneven sequencing coverage; or 2) MITObim (Hahn et al. 2013) for challenging
243 assemblies through the provision of bait sequences to recruit reads for more localised
244 assemblies of the mitogenome or specific nuclear genes.
245 Target sequences were identified from these assemblies through sequence type
246 specific methods (e.g. mitochondrial, nuclear protein-coding genes, nuclear ribosomal
247 RNA genes). Complete mitochondrial sequences were recovered for most samples
248 from either *de novo* (IDBA-UD) or baited (MITObim) assemblies and annotated with
249 MITOS (Bernt et al. 2013). Any recalcitrant gaps (i.e. more than one contig) were
250 gap-closed through PCR using gap-bridging primers and Sanger sequencing. Nuclear
251 ribosomal RNAs were predicted with RNAmmer (Lagesen et al. 2007) and the
252 nuclear protein-coding gene (histone H3) was recovered through a BLASTn search
253 (Altschul et al. 1990) against existing H3 sequences of related species. For histone
254 H3, the start and stop coordinates were further refined with ORF Finder
255 (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and translated with the *transeq*
256 component provided by EMBOSS (Rice et al. 2000) to obtain the amino acid
257 sequence.
258 The same genome skimming workflow was tested on reads sequenced from
259 species representing a diversity of animal phyla, including representatives of the
260 Mammalia, Arthropoda, Aves, Teleostei and Mollusca to evaluate the general
261 applicability of our methods across different animal groups and tissue types.
262 Specifically, sequence reads were obtained from nine other sequencing projects in our
263 laboratory and three projects on NCBI's SRA database for species from a variety of
264 animal Phyla and Classes and tissue sources (e.g. fin clips, liver, muscle, whole

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265 organism). These sequence datasets were inspected for the presence of reads for the
266 same three nuclear genes (18S, 28S, H3) recovered from this study of crayfish and
267 lobster species.
268
269 *Phylogenetic analyses*
270
271 The construction of phylogenetic trees was carried out on seven different
272 alignments (Datasets A to G, Table 1), consisting of various combinations of genes,
273 sequence types (amino acid, aa, vs nucleotide, nt) and lengths. In analyses that utilise
274 only mitochondrial gene sequences (13 protein-coding genes, 2 rRNAs), a total of 33
275 samples from the families Astacidae (7), Cambaridae (16), Parastacidae (6) and
276 Nephropidae (4) were included. Datasets that included the nuclear genes (18S rRNA,
277 28S rRNA and histone H3) sampled fewer taxa (24), subject to the availability of
278 these gene sequences on NCBI for species that were not sequenced in our laboratory
279 (e.g. *Procambarus alleni, Procambarus fallax*).
280 Amino acid sequences of protein-coding genes (mitochondrial, H3) as well as
281 nucleotide sequences of non-coding rRNAs (12S, 16S, 18S, 28S) were aligned with
282 MAFFT (*mafft-linsi*) (Katoh & Standley 2013) and trimmed with trimAl
283 (*automated1*) (Capella-Gutiérrez et al. 2009). Nucleotide sequences of protein-coding
284 genes were aligned with TranslatorX (Abascal et al. 2010), which carries out
285 nucleotide sequence alignment guided by amino acid translations followed by
286 alignment trimming with Gblocks (Castresana 2000) implemented internally by the
287 same program.
288 For phylogenetic analyses, alignments were concatenated for each of the seven
289 datasets (Table 1) and supplied as partitioned alignments to IQ-TREE (Nguyen et al.
290 2014) for model testing and maximum-likelihood analysis, with node supports
291 obtained with the Ultrafast bootstrap option (Minh et al. 2013). The same partitioned
292 alignments were used for Bayesian inference using ExaBayes (Aberer et al. 2014).
293 Four independent chains were run for a minimum of 5 million generations each, with
294 25% of initial samples as burn-in, and convergence of chains was determined when
295 the average standard deviation of split frequencies (*asdsf*) fell below 1% indicating
296 good convergence.
297
298 *Topology testing*

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299
300 Topology testing was carried out using IQ-TREE (Nguyen et al. 2014) to evaluate the
301 likelihood of alternate topologies (e.g. a monophyletic Cambaridae). The following
302 tree topology tests were performed using Dataset F (Table 1), comprised of 18 genes
303 (13 mitochondrial PCGs, 12S, 16S, 18S, 28S rRNA, H3):
304
305 I: ((Astacidae, Cambaridae-NA), Cambaridae-Asia), outgroups)
306 II: (Astacidae, (Cambaridae-NA, Cambaridae-Asia)), outgroups)
307 III: ((Astacidae, Cambaridae-Asia), Cambaridae-NA, outgroups)
308
309 The tree topology tests include the Shimodaira-Hasegawa test (Shimodaira &
310 Hasegawa 1999) carried out using the RELL approximation (Kishino et al. 1990)
311 based on 1,000 replicates and the approximately unbiased (AU) test (Shimodaira
312 2002).
313
314 **Results**
315
316 *Genome skimming effectively recovers the mitogenome sequence and high copy*
317 *number nuclear genes*
318
319 An average of approximately 739.4 Mbp of raw sequence data per sample was
320 generated from the freshwater crayfish and lobster libraries (Supplementary Data 1).
321 Mitogenome sequences assembled for these species vary in size from 14,895 bp to
322 20,677 bp with AT content ranging from 67.9% to 73.1%. The typical 13
323 mitochondrial protein-coding genes, 22 transfer RNA genes and 2 ribosomal RNA
324 genes (12S, 16S) are found in all Northern Hemisphere crayfish and lobster
325 mitogenomes recovered in this study (marked with '*' in Supplementary Data 1). The
326 organization of these genes in the mitogenomes of the ten Northern Hemisphere
327 crayfish taxa we assembled is identical to the first sequenced species, *Procambarus*
328 *clarkii*, which itself shows a large departure from the ground pancrustacean pattern
329 (as represented by *Drosophila*, *Penaeus monodon* and the outgroup species, *Homarus*
330 *americanus*). The gene order for the lobster *Metanephrops sibogae* is also aberrant
331 compared to *H. americanus*, a result of multiple translocated protein-coding and
332 tRNA genes. Most notably, *M. sibogae* possesses two control regions, each

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333 approximately 2 kbp in length, resulting in a much longer mitochondrial genome size
334 of 20,677 bp. The lengths of the 12S and 16S rRNA genes are generally shorter in the
335 Northern Hemisphere crayfish compared to the Southern Hemisphere crayfish.
336 Details such as coding regions, AT-content and intergenic lengths for each
337 mitogenome are available in Supplementary Data 3.

338 Complete or near-complete sequences were recovered for the three nuclear
339 genes 28S rRNA (4,144 - 5,391 bp), 18S rRNA (1,869 - 1,885 bp) and H3 (all 411
340 bp) from the same partial genome scan. Their degrees of similarity to available
341 sequences on NCBI for the same species are detailed in Supplementary Data 4. Out of
342 the total nuclear sequences contributed through this study, 38 gene sequences from 13
343 species are 'novel' (i.e. do not have any representation on NCBI). The remaining 28
344 gene sequences are highly similar to sequences held on NCBI based on local
345 alignment, with average percent identities of 98.7% (28S), 99.4% (18S) and 98.9%
346 (H3) for matching species. The lengths of the 18S rRNA sequences recovered in this
347 study are comparable to those already available on public databases through PCR-
348 based methods. However, the other nuclear sequences (28S, H3) obtained from
349 genome skimming are much longer in length than those deposited on NCBI for
350 crayfish and lobster species. Notably, the 28S rRNA gene sequences contributed in
351 this study are almost double the length of their same-species counterpart available on
352 NCBI. Also, the full length amino acid sequence of the histone H3 gene (411 bp)
353 complete with start and stop codons was recovered, as opposed to the currently
354 available partial H3 sequences that are mostly 333 bp or shorter. All recovered
355 mitochondrial and nuclear sequences are available on NCBI at accession numbers
356 listed in Supplementary Data 1.
357
358 *Phylogenetic analyses and topology tests point to a polyphyletic Cambaridae*
359
360 The nucleotide-based phylogenetic tree was generated from the longest
361 alignment (16,211 bp, Dataset F; Fig. 2) with representative species from
362 Parastacoidea (Southern Hemisphere crayfish) and Nephropoidea (lobsters) as
363 outgroups. The focus of this study, the superfamily Astacoidea, is represented by
364 species from two families, Astacidae (5 species) and Cambaridae (9 species).
365 Maximal support is observed for most nodes in this clade of interest, except for the
366 weaker ML support for the sister relationship between astacids and North American

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367 cambarids (ultrafast bootstrap: 86, PP: 1.00). Both ML and BI trees inferred from this
368 dataset imply a monophyletic Astacidae and a polyphyletic Cambaridae, with the split
369 occurring between the North American cambarids (*Procambarus*, *Cambarus*,
370 *Orconectes*) and the Asian cambarids (*Cambaroides*). Further topology testing (Fig.
371 3) shows Topology I (North American cambarids as sister taxa to astacids) as most
372 likely, followed by Topology III (Asian cambarids as sister taxa to astacids). Both
373 Topology I and Topology III support a polyphyletic Cambaridae. Topology II,
374 containing a monophyletic Cambaridae, is rejected (P-value < 0.05 for tree tests in
375 Fig. 3).

376 Nevertheless, tree topologies are variable depending on the dataset and the
377 method used to infer phylogeny (Fig. 4A-G). While the most common topology is
378 consistent with the tree in Fig. 2, other observed topologies mostly differ in the
379 relationships among groups of the North American cambarids (Datasets B and C).
380 The tree generated from Dataset G, which consists of only the nuclear 18S, 28S and
381 H3 gene sequences (4,205 aligned sites), deviates from the other topologies. Its
382 Bayesian tree does show a monophyletic Cambaridae but with only weak support (PP:
383 0.61), and is incongruent with the ML tree generated from the same alignment, which
384 is similar to the other analyses and also fails to recover a monophyletic Cambaridae.
385 Detailed ML and BI phylogenetic trees inferred from all datasets are available as
386 Supplementary Data 5.

387

388 *Generality of genome skimming for nuclear genes for animals*

389

390 Out of the twelve tested animals, the 18S gene sequence was recovered from
391 all species, whereas sequences from both 28S (partial or complete) and H3 were
392 recovered from ten out of twelve species. Similarly, genome skimming successfully
393 recovered substantially longer 28S (approximately 4 kbp) and H3 (411 bp) gene
394 sequences in most cases compared to sequences available on NCBI (28S: 1.5 - 4 kbp,
395 H3: 333 - 411 bp; Supplementary Data 4). The 18S, 28S and H3 sequences recovered
396 for these species are available as Supplementary Data 6.

397

398 **Discussion**

399

400 *Crayfish mitogenomes*

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401

402 This study increases the number of sequenced Northern Hemisphere crayfish
403 mitogenomes from six to sixteen, substantially expanding the available resources for
404 the family Cambaridae (*Procambarus*, *Cambaroides, Cambarus, Orconectes*) and
405 Astacidae (*Astacus*, *Pacifastacus, Austropotamobius*). In addition, a new mitogenome
406 for the lobster, *Metanephrops sibogae*, reveals an aberrant gene order for this group,
407 but one identical to that recently described for *Metanephrops thomsoni* (Ahn et al.
408 2016). This is a surprising finding given that previous studies indicated that marine
409 lobsters (*Homarus americanus* and *Enoplometopus*) possess a conserved mitogenome
410 order that is common across the arthropods and is considered reflective of the
411 primitive pancrustacean pattern (Boore et al. 1995; Shen et al. 2013).
412 Another equally surprising finding is the lack of mitogenome variation among
413 Northern Hemisphere species, given the high frequency of novel mitogenome gene
414 orders among Southern Hemisphere crayfish. No mitogenome gene rearrangements
415 are apparent for the ten new mitogenomes provided from this study and the six from
416 previous studies, all of which contributes to taxonomic sampling covering all families
417 and the full geographic range of the superfamily. This is in stark contrast to the
418 number and scale of mitogenome gene order rearrangements among Southern
419 Hemisphere crayfish with most genera studied having distinct gene orders, including
420 interspecific differences within the genus *Engaeus* (Lee et al. 2016; Tan et al. 2015).
421 The frequency of mitogenome rearrangements is not simply a function of
422 divergence times. Based on the dated phylogeny of Bracken-Grissom et al. (2014), the
423 *Engaeus* group of crayfish and its close relatives, containing significant
424 rearrangements, diverged more recently (145.4 mya) than the Northern Hemisphere
425 crayfish as a group (161.2 mya), which have none. Conversely, *Euastacus* and
426 *Cherax*, which last shared a common ancestor approximately 200 mya have identical
427 mitogenome gene orders. Thus, crayfish exhibit both extreme conservation and
428 extreme lability of mitochondrial gene order, that is not a simple function of
429 divergence time, an observation that invites further investigation on the dynamics and
430 evolutionary drivers of mitogenome evolution in this group (Kilpert et al. 2012;
431 Okajima & Kumazawa 2010; Poulsen et al. 2013).

432

433 *Phylogenetic results and the status of the family Cambaridae*

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435 This study contributes to growing evidence suggesting that the family
436 Cambaridae is non-monophyletic, but contradicts suggestions that the genus
437 *Cambaroides* should be included within the family Astacidae. Several studies using a
438 variety of morphological and molecular datasets from a range of genes and varying
439 taxonomic sampling concur that North American cambarid species and Asian
440 cambarid species (genus *Cambaroides*) do not share a common ancestor (Ahn et al.
441 2006; Braband et al. 2006; Bracken-Grissom et al. 2014; Bracken et al. 2009;
442 Breinholt et al. 2009; Crandall et al. 2000; Porter et al. 2005; Rode & Babcock 2003).
443 The phylogenetic position of Asian cambarid species and their taxonomic treatment
444 within the superfamily Astacoidea remains controversial.
445 While most studies have supported the Asian cambarid lineage as the most
446 basal within the Astacoidea, Bracken-Grissom et al. (2014) found the Asian
447 cambarids and the astacids to be monophyletic (using a combination of morphological
448 characters, three mitochondrial and three nuclear gene fragments and based on two
449 samples of *Cambaroides japonicus*, single samples of *Astacus astacus* and
450 *Austropotamobius torrentium,* and four samples of *Pacifastacus*). They suggested that
451 the concept of the Astacidae should be expanded to include *Cambaroides*. Instead,
452 our dataset strongly supports the *Cambaroides* lineage to be basal, based on our data
453 from five astacid species, two *Procambarus*, two *Orconectes*, one *Cambarus* and four
454 *Cambaroides* species consisting of both nuclear and mitochondrial genes.
455 A basal position for the Asian cambarid lineage requires a re-evaluation or re-
456 interpretation of morphological and reproduction-related characters as either ancestral
457 or convergent within the lineages as recovered in this study (Ahn et al. 2006; Braband
458 et al. 2006). We suggest a family-level revision of the taxonomic classification of
459 Northern Hemisphere crayfish that might consider placing the Asian cambarid
460 crayfish in a new family, or placing all Northern Hemisphere crayfish in a single
461 family, similar to the treatment of all Southern Hemisphere crayfish as members of
462 the Parastacidae.
463
464 *The utility of genome skimming for animal phylogenetics*
465
466 This study demonstrates the utility of partial genome sequencing, also known
467 as genome skimming, using the MiSeq NGS platform as a rapid and inexpensive
468 approach to assemble substantial datasets to support phylogenetic studies. We used

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469 our crayfish dataset to construct an alignment of 12,006 nucleotides from the
470 mitochondrial genomes, which is now becoming a routine procedure for animal
471 phylogenetic studies using NGS (Gan et al. 2014; Shen et al. 2013; Tan et al. 2015).
472 Less common is the use of sequences from nuclear genes that can also be
473 recovered from the same partial genome scan used to assemble whole mitogenome
474 sequences or to locate microsatellite markers for population genetic applications (Gan
475 et al. 2014; Thai et al. 2016). Supplementary Data 7 summarises the only four recent
476 studies we could find on animals that have reported nuclear genes recovered from
477 NGS-based genome scans (Besnard et al. 2016; Kocher et al. 2015; Kocher et al.
478 2014; Richter et al. 2015). Genes and regions associated with the nuclear ribosomal
479 cluster are the most common target, and these studies together with our data indicate
480 that complete or almost complete gene sequences can be routinely recovered for the
481 18S and 28S genes from various animal groups including annelids, crustaceans,
482 molluscs (Bivalvia and Gastropoda), and chordates (Aves, Chondrichthyes,
483 Actinopterygii, Mammalia). Further, high copy number protein-coding genes can also
484 be recovered. Our study is the first to report recovery of the histone H3 gene, for
485 which the full amino acid sequence was retrieved for all our lobster and crayfish
486 samples and ten of twelve species in our supplementary non-crustacean datasets
487 (Table 2). It was also encouraging that other protein-coding genes can potentially be
488 recovered from shotgun sequencing datasets (Besnard et al. 2016), especially as the
489 phylogenetic utility of ribosomal nuclear genes has been called into question by some
490 authors (Tsang et al. 2008).
491 We foresee exciting times ahead for the discovery and recovery of an
492 increasing number of nuclear genes for phylogenetic analyses, given increasing use of
493 NGS for partial genome sequencing for many animal samples plus the increasing
494 number of whole genome sequences becoming available for a diversity of animal
495 species. Further, we anticipate that animal systematics is entering a new era in which
496 even more robust datasets can be assembled, maximising both taxon and gene
497 sampling while minimising expense to an extent hitherto impossible (Richter et al.
498 2015; Straub et al. 2012).
499

500 **Declaration of Interest**

501

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502 The authors report no conflicts of interest and the authors alone are responsible for the
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504
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774 **Figure legends**

775

776 **Fig. 1.** Genome skimming workflow used to recover the mitogenome and high copy
777 number nuclear genes from partial genome scans.

778

779 **Fig. 2.** Phylogenetic relationships among Northern Hemisphere freshwater crayfish
780 inferred based on nucleotide alignment of Dataset F (Table 1) comprising of 13
781 mitochondrial PCG, 12S, 16S, 18S, 28S and H3 (16,211 sites). Topology shown was
782 obtained from Bayesian inference, with Ultrafast bootstrap values (from Maximum
783 likelihood analysis) and posterior probabilities indicated as support values at each
784 node. Square brackets '[ ]' indicate conflict in topology inferred by the two
785 phylogenetic methods.

786

787 **Fig. 3.** Evaluation of alternate tree topologies through topology testing based on
788 Dataset F (13 mt-pcg (nt) + 12S + 16S + 18S + 28S + H3).

789

790 **Fig. 4.** An overview of evolutionary relationships within Astacoidea (outgroups:
791 Parastacoidea and Nephropoidea). Tree topologies were constructed from each of the
792 seven datasets (Table 1) and numbers at the upper left corner of each tree indicate
793 dataset used for phylogenetic inference. Ultrafast bootstrap and/or posterior
794 probability values are used to show support at each node while coloured branches
795 highlight differences in topology between ML (left) and BI (right) trees.

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Table 1. Datasets used to construct alignments used in phylogenetic analyses. Trees inferred from these datasets are available in Supplementary Data 5.

Dataset	# Taxa	# Genes	Genes included	Alignment length (sites)
A	33	13	mt-pcg (aa)	3,657
B	33	15	mt-pcg (aa) + 12S + 16S	5,254
C	24	18	mt-pcg (aa) + 12S + 16S + 18S + 28S + H3	9,459
D	33	13	mt-pcg (nt)	10,449
E	33	15	mt-pcg (nt) + 12S + 16S	12,006
F	24	18	mt-pcg (nt) + 12S + 16S + 18S + 28S + H3	16,211
G	24	3	18S + 28S + H3	4,205

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Table 2. Demonstration of the recovery of 28S rRNA, 18S rRNA and histone H3 sequences from performing genome skimming on sequence reads of animals from various taxonomic groups and tissue isolation sources. Gene sequences recovered for these animals are available in Supplementary Data 6.

Phylum	Class	Species	Tissue source	Sequence data	Recovered gene length (bp)		
					28S	18S	H3
Chordata	Actinopterygii	Gadopsis marmoratus[a]	fin clip	459 Mb	4492	1840	411
		Oryzias latipes[b]	SRA	1 Gb	4720	1842	411
	Aves	Corvus splendens[c]	liver	813 Mb	-	1822	411
	Chondrichthyes	Pastinachus atrus[d]	muscle	3.45 Gb	2699	1796	411
	Mammalia	Gallus gallus[e]	SRA	2 Gb	2065	1822	411
		Rattus norvegicus[f]	SRA	2 Gb	4803	1871	411
Mollusca	Bivalvia	Lutraria rhynchaena[g]	muscle	623 Mb	4201	1839	411
		Tridacna squamosa[h]	muscle	203 Mb	4314	1870	411
	Gastropoda	Babylonia areolata	muscle	61 Mb	4394	1828	411
Arthropoda	Branchiopoda	Triops australiensis[i]	whole	920 Mb	3988	1810	-
	Maxillopoda	Lepas anserifera	whole	425 Mb	4125	1870	411
		Pandarus rhincodonicus[j]	whole	480 Mb	-	1814	-

Raw reads were obtained from various internal projects and databases: a. Gan et al. (2016c); b. ERR110365 (SRA); c. Krzeminska et al. (2016); d. Austin et al. (2016a); e. SRR2131206 (SRA); f. ERR316506 (SRA); g. Gan et al. (2016d); h. Gan et al. (2016a); i. Gan et al. (2016b); j. Austin et al. (2016b).

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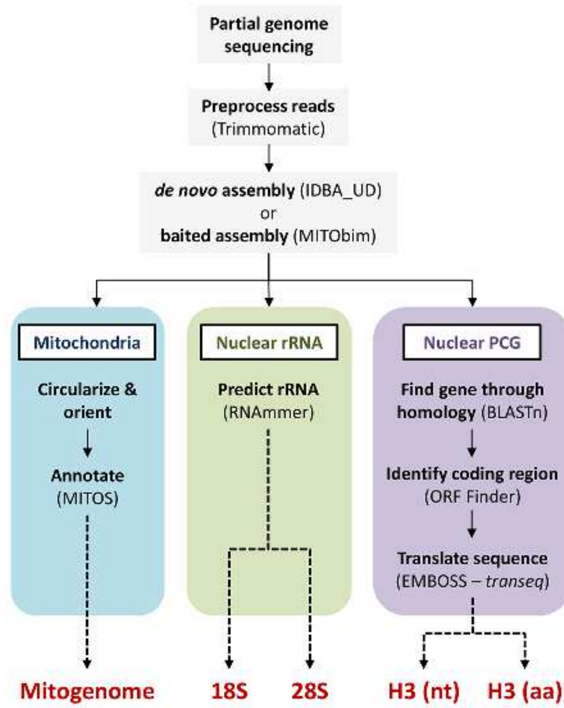

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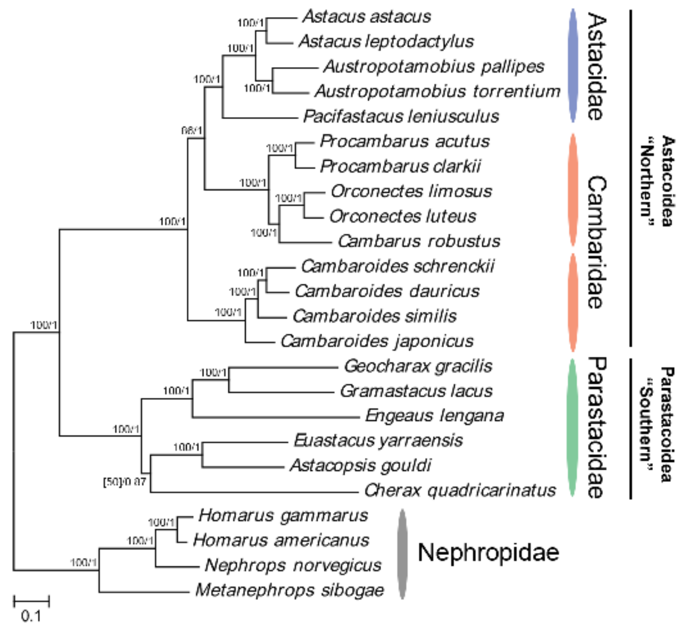

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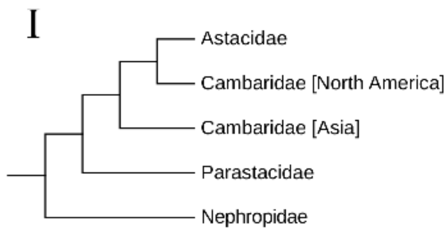

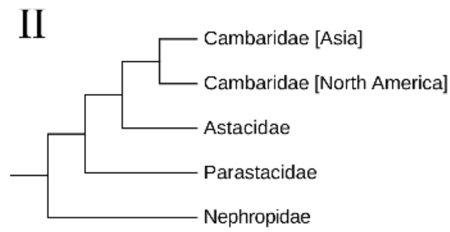

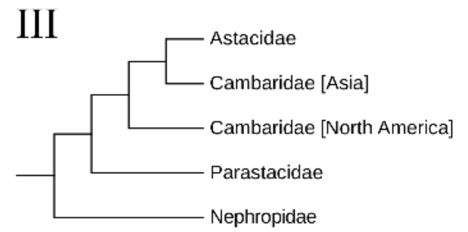

Tree	logL[1]	p-SH[2]	p-AU[3]
I	-155582.703	1.0000 (+)	0.5227 (+)
II	-155610.006	0.0350 (-)	0.0176 (-)
III	-155593.224	0.3300 (+)	0.2577 (+)

Plus signs (+) denote the 95% confidence sets
Minus signs (-) denote significant exclusion

[1] Tree log-likelihood
[2] P-value of Shimodaira-Hasegawa test (2000)
[3] P-value of approximately unbiased test (Shimodaira, 2002)

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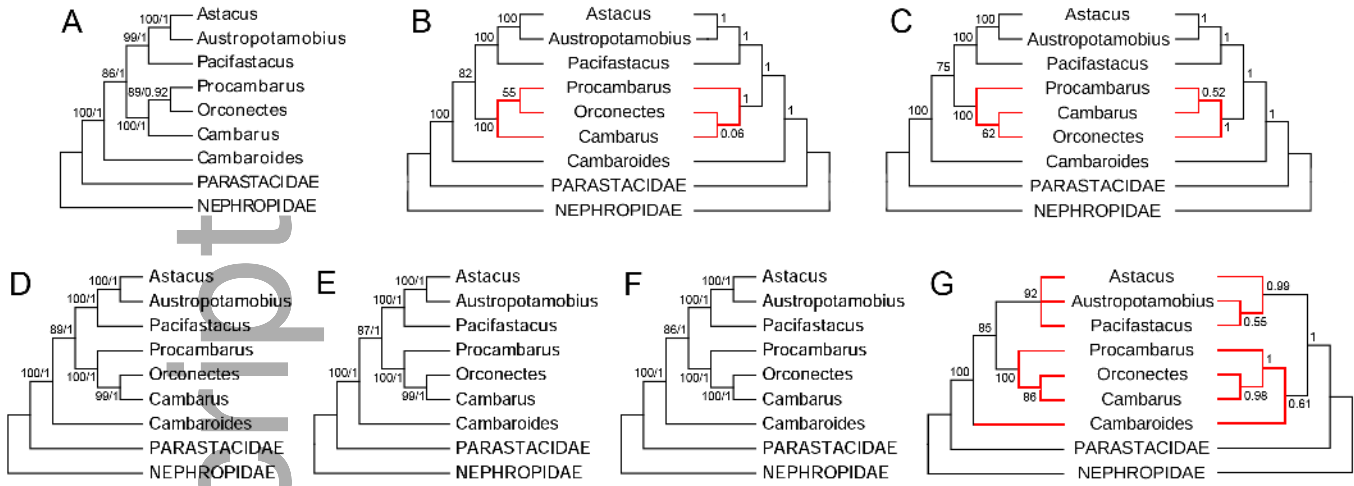

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