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**Impact of transposable elements on genome size variation between
two closely related crustacean species**

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Abstract

Identifying and quantifying genome size variation among species and understanding if it evolves as a consequence of adaptive or stochastic processes is still a long-standing objective in evolutionary biology. Here, we investigated the basis of genome size variation between two closely related species of terrestrial isopods: *Armadillidium vulgare* and *Armadillidium nasatum* (Crustacea, Oniscidea). The two species diverged 25 million years ago and they substantially diverge in genome size, the *A. vulgare* genome being ~500 megabases larger than the *A. nasatum* genome (1.7 vs. 1.2 gigabases, respectively). Our analyses indicated that genome size difference is essentially attributed to transposable elements (TEs). As the two genomes largely share the same TE families, differential transpositional activity likely underlies the observed variation in TE copy numbers and genome size. Analyses of TE expression suggested that there may be ~4 times more transcribed TE copies in *A. vulgare* than in *A. nasatum*. However, the cumulative expression level of all expressed TEs was higher in *A. nasatum* than in *A. vulgare*, suggesting that the two species may have recently been experiencing different TE transposition dynamics. Overall, our results illustrate the important impact TEs can have on genome structure and evolution between closely related species.

Keywords

transposable elements, genome sequencing, comparative analysis, crustaceans, terrestrial isopods, genome size

1. Introduction

Genome size variation is one of the most noteworthy sources of biodiversity on Earth [1]. The nature and importance of the evolutionary forces and the molecular mechanisms causing this variation are the subject of numerous studies and debates [2–8]. Many studies have shown that genome size is positively correlated to the content in transposable elements (TEs), as for example in mammals [9], birds [9], insects [10–16], or flowering plants [17,18]. However, the reasons explaining why TEs accumulate more in some species than in others remain largely unknown.

To date, more than 4,000 genomes of Eukaryota have been sequenced and are available in GenBank (last accessed on December 2019). Crustaceans, however, are poorly represented among these genomes. Despite the existence of more than 70,000 living species of crustaceans [19], the genomes of only 30 species are available to date. While crustaceans are primarily marine organisms, constituting a large proportion of ocean biomass [20], many species have colonized freshwater, semi-terrestrial or terrestrial environments. Recently, the genomes of two species of terrestrial isopods have been assembled : *Armadillidium vulgare* [21] and *Armadillidium nasatum* [22]. These two species are closely related and they diverged from a common ancestor ~25 million years ago [23]. However, it turns out that the genome assembly of *A. vulgare* (1.7 Gb) [21] is ~500 megabases larger than that of *A. nasatum* (1.2 Gb) [22]. In both cases, the genomes were sequenced using the same hybrid strategy combining Illumina short reads and PacBio long reads, and following the same assembly protocol [21,22]. These resources offer an interesting opportunity to compare genome size evolution between two closely related species. In this study, we used a comparative approach to investigate the causes of the large genome size variation observed between the two closely related species of terrestrial isopods *A. vulgare* and *A. nasatum*. Our results indicate that differential transpositional activity of the same TE families likely underlies most of the variation in genome size between the two species.

2. Materials and Methods

2.1 Genomic data

The genomes of *A. vulgare* (accession number : SAUD000000000) and *A. nasatum* (accession number : SEYY000000000) were both assembled using a hybrid approach combining short paired-end Illumina reads and long PacBio reads and the exact same suite of software, as described previously [21] [22]. The *A. vulgare* assembly is composed of 43,541 contigs/scaffolds, totaling 1.725 Gb with an N₅₀ (minimum contig length to cover 50% of the genome) of 51,088 bp. Based on a BUSCO analysis, we found that it includes 92.0% of the 1,066 specific arthropod core genes [24]. The *A. nasatum* assembly is composed of 25,196 contigs/scaffolds, totaling 1.223 Gb with an N₅₀ of 86,284 bp and including 93.9% of the 1,066 specific arthropod core genes.

2.2 Comparison of orthologous genes

To identify orthologous genes between *A. vulgare* and *A. nasatum* genome assemblies, protein sequences were obtained for the two species based on the structural and functional annotation of both assemblies and they were compared using the ortholog research tool OrthoMCL [25]. In total, 19,051 and 14,636 proteins were used for *A. vulgare* and *A. nasatum*, respectively. The BLASTP E-value cutoff of the OrthoMCL pipeline was set to 10^{-10} .

2.3 Analyses of TE expression

To identify the genomic origin of expressed TEs, we used the pipeline TEcandidates [26]. In brief, TEcandidates uses *de novo* transcriptome assembly to assess expressed TE instances. To restrict reads to repeat zones, TEcandidates first runs a pre-alignment of the reads on the genome and keeps the reads that map to RepeatMasker predictions. Then, a *de novo* transcriptome assembly is performed using Trinity v2.4.0 with default parameters [27]. Generated contigs are mapped to the reference genome, and their intersection with

annotated TEs is assessed with BEDtools v2.26 [28]. We used the multifasta files corresponding to *A. vulgare* and *A. nasatum* genomes, the annotation gff files generated by RepeatMasker (as each genome annotation has resolution at the level of TE instance), and RNAseq datasets generated previously (100 bp-long single end reads) [29]: 13 datasets for *A. vulgare* (SRA accession number : SRX564995 to SRX565004) and two for *A. nasatum* (SRX564993 and SRX564994). TEcandidates pipeline was then used with default parameters, as recommended by the authors instructions. RNAseq read quality was analyzed with FastQC (version 0.11.4, <http://www.bioinformatics.babraham.ac.uk/projects/89fastqc>). Removal of low-quality reads and sequencing adaptors was performed with Trimmomatic (version 0.33), setting the ILLUMINACLIP palindrome clip threshold at 30 and the simple clip threshold at 10. The quality threshold was set to a minimum Phred score of 20.

To quantify TE expression, we mapped the RNAseq reads onto the potentially expressed TEs identified by TEcandidates. Reads from multiple RNAseq datasets were mapped independently using Bowtie2 [30] using the *very sensitive* mode (parameter : *end-to-end*). TE genomic coverage was calculated using BEDTools [28], and the Artemis software (v18.0.0) [31] was used to quantify expressed transcripts, and yielded reads per kilobase of exon model per million mapped (RPKM) expression values.

3. Results & Discussion

3.1 Gene content

To investigate the possible factors underlying the difference in genome size between *A. vulgare* and *A. nasatum*, we first compared the gene content of the two species. The structural annotation identified 19,051 and 14,636 genes in *A. vulgare* and *A. nasatum*, respectively. The large difference in gene number between the two genomes (4,415) corresponds to 26.6 Mb, as all genes combined represent 164.5 Mb in *A. vulgare* [21] and 137.9 Mb in *A. nasatum* [22]. Thus, variation in gene number only accounts for 5% of the genome size difference between the two species.

To investigate the possible causes underlying the difference in gene number between the two species, all proteins annotated from the genomes of *A. vulgare* and *A. nasatum* were classified by OrthoMCL in 9,140 groups of orthologous proteins (orthogroups). Among them, 8,036 orthogroups contained sequences belonging to both *A. vulgare* and *A. nasatum*, for a total of 10,815 and 9,429 proteins (Figure 1, Table 1). The OrthoMCL analysis further identified 8,236 and 5,207 genes specific to *A. vulgare* and *A. nasatum*, respectively (Figure 1), of which 6,390 and 4,226, respectively, are present as single-copy genes (Table 1).

As both species appear to exhibit high numbers of specific genes, it is possible that some of them are artifacts of the annotation process. Indeed, a higher proportion of genes with unknown function is found among species-specific genes relative to genes common to both species (Table 1). It is conceivable that some species-specific genes may in fact be TE genes that would have been misidentified and mistakenly attributed to non-TE genes (without known function), and this may have affected *A. vulgare* to a larger extent than *A. nasatum* because both species differ in their TE content (see section 3.2 below). In addition, our results suggest that after the divergence between the two terrestrial isopods, the genome of *A. vulgare* has undergone more gene duplications than that of *A. nasatum* (Figure 1). Gene duplication is a major force behind biological innovation and is often associated with speciation phenomena [32]. This innovative force has been characterized many times in the plant kingdom [33–36] as well as in animals [37,38]. Thus, the difference in gene number might have contributed to the divergence between *A. vulgare* and *A. nasatum*. As it has the potential to increase phenotypic plasticity [39], gene duplication may confer a better adaptive potential, particularly during the colonization of new environments, as recently demonstrated in aphids [40]. However, it is unclear if such a hypothesis may hold for *A. vulgare* relative to *A. nasatum*, because both species are known to share the same distribution areas, and they are therefore potentially subject to similar environmental constraints [41]. In sum, while further investigations are needed to better understand the evolutionary basis of gene number variation in the two terrestrial isopod species, this parameter does not explain the difference in genome size between *A. vulgare* and *A. nasatum*.

3.2 Repeat content

Comparison of tandem repeat content between *A. vulgare* and *A. nasatum* indicated similarly high amounts: 303.6 Mb (or 17.6% of the genome) for *A. vulgare* and 285.4 Mb (or 23.3% of the genome) for *A. nasatum* (Figure 2, Table 2). Thus, tandem repeats do not contribute much to the difference in genome size between *A. vulgare* and *A. nasatum*. In fact, the difference in size between the two genomes is mainly explained by a much larger number of TE copies (~0.6 million) in *A. vulgare* relative to *A. nasatum*. Indeed, there are ~2.1 million TE copies (or fragments) annotated by RepeatMasker in *A. vulgare* vs. only ~1.5 million in *A. nasatum* (Table 2). Consequently, the genome of *A. vulgare* has 854.1 Mb derived from TEs while the genome of *A. nasatum* has only 484.7 Mb (Figure 2, Figure 3, Table 2). Thus, 74% of the observed size difference between the two genomes is accounted for by TEs (Table 2).

Interestingly, the activity peak of TEs in *A. nasatum* corresponds to copies of TEs with ~10% divergence from their consensus sequence, while the activity peak of TEs in *A. vulgare* corresponds to copies of TEs with ~6% divergence from their consensus sequence (Figure 3). The age profile thus suggests that *A. vulgare* underwent a peak in transpositional activity at about the time of or not long after the divergence between *A. vulgare* and *A. nasatum* (estimated to ~25 million years ago or ~6% divergence [23]). This is likely to explain the difference in TE abundance between the two genomes. The alternative explanation is that *A. nasatum* has a higher deletion rate compared to *A. vulgare*. Unfortunately, no outgroup is available for testing this hypothesis. In the absence of mechanisms known to specifically delete TEs, the most plausible explanation for the observed genome size differences currently remains that of a higher transpositional activity in *A. vulgare* than in *A. nasatum*.

According to our analyses, the most represented TEs in both species belong to the LINE category (accounting for >20% of each genome, Figure 2). These LINEs are divided into 14 families shared by both species (Figure 3). The second most represented TEs are DNA transposons, which represent 16.2% of the *A. vulgare* genome and 12.8% of the *A. nasatum* genome (Figure 2). These DNA transposons belong to 17 families shared by both genomes (Figure 3). The third most represented TEs are LTR retrotransposons which represent 5-8% of

both genomes (Figure 2), distributed in 10 families shared by both species (Figure 3). In sum, the difference in TE numbers between *A. vulgare* and *A. nasatum* is not due to the acquisition and expansion of new TE families in *A. vulgare* relative to *A. nasatum*. Instead, differential amplification of the same TE families appears to underly variation in TE content between the two species.

In line with a growing body of literature derived from diverse taxonomic groups ranging from vertebrates [9,42,43] to invertebrates [11–14,44] and plants [45–47], our analysis shows that genome size variation across two closely related crustacean species can largely be explained by differences in TE content. As the divergence time between *A. vulgare* and *A. nasatum* is estimated to ~25 million years and genome size difference due to TE abundance is roughly 370 Mb (Table 2), genome size divergence between *A. vulgare* and *A. nasatum* is estimated to ~15 Mb per million years. Considering genome sizes in the gigabase range, this rate may not seem stunning, but it appears to be strong enough to generate substantial genome size differences at an evolutionary time scale.

3.3 Comparison of TE expression profiles in *A. vulgare* and *A. nasatum*

As the larger genome size of *A. vulgare* is likely explained by a higher rate of TE amplification since the last common ancestor it shares with *A. nasatum*, we assessed whether this higher amplification rate was associated with higher levels of TE expression. The TE candidate pipeline identified 5,317 TE copies (distributed in 4,055 different contigs) potentially transcribed in *A. vulgare* (0.25% of the total number of TE copies) compared to 1,259 in *A. nasatum* (0.08% of the total number of TE copies, distributed in 1,101 genomic contigs) (Table 3). This analysis suggested that there may be ~4 times more transcribed TE copies in *A. vulgare* than in *A. nasatum*. In both species, LINEs were the TEs with the highest number of transcribed copies TEs, comprising >50% of all transcribed copies, whereas DNA transposons accounted for ~25% of all transcribed copies (Table 3).

To quantify TE expression levels in the two genomes, we carried out RPKM analyses based on RNAseq data. It appears that the most highly expressed TEs in *A. vulgare* and *A.*

nasatum are different. Indeed, CR1-Zenon LINEs are by far the most highly expressed TEs in *A. vulgare*, followed by unclassified DNA elements, unclassified LINEs, RTE-BovB LINEs and Gypsy LTR retrotransposons (Figure 4). In contrast, unclassified DNA transposons are by far the most highly expressed TEs in *A. nasatum*, followed by CR1-Zenon LINEs, Gypsy LTR retrotransposons RTE-BovB LINEs and unclassified LINEs (Figure 4). Interestingly, the cumulative expression level of all expressed TEs was higher in *A. nasatum* than in *A. vulgare*, with total cumulative RPKM values of 778,709 and 686,967, respectively. Thus, our results suggest that the species with the lowest, not the highest, TE amplification rate is associated with the highest TE expression level. Assuming that TE expression level is a good proxy for TE transpositional activity, our results may reflect that TE transposition rate is varying in opposite directions in the two species: it may be declining in *A. vulgare* after a phase of intense amplification while it may be rising or steady in *A. nasatum*. In any event, identification of cases of TE horizontal transfer during the recent evolutionary history of *A. vulgare* [48] supports recent transpositional activity in this species, although further investigations will be needed to evaluate its magnitude.

4. Conclusion

In summary, our analyses demonstrated that even though the *A. vulgare* genome contains almost 4,500 more annotated genes than that of *A. nasatum*, this difference does not contribute much to the observed difference in the genome size of *A. vulgare* relative to *A. nasatum*. Instead, the large difference in genome size (~500 megabases) between the two closely related terrestrial isopods *A. vulgare* and *A. nasatum* can essentially be explained by a difference in TE content likely caused by a higher transposition activity in *A. vulgare* than in *A. nasatum*, subsequent to the divergence between the two species ~25 million years ago. Differential transpositional activity of the same TE families apparently underlies the variation in TE copy numbers and genome size between the two species. Our results highlight the tremendous impact of TEs on the structure and evolution of genomes previously observed in a broad range of organisms, and extended here to two closely related crustacean species.

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248

249 **Authors Contributions:**

250 Conceptualization: T.B, C.G. and R.C.; Funding acquisition: R.C; Methodology: T.B, C.G. and
251 R.C; Supervision: C.G. and R.C.; Visualization: T.B; Writing – Writing – Original Draft
252 Preparation: T.B, C.G. and R.C.; Writing – Review & Editing: T.B, C.G. and R.C.

253

254 **Conflicts of Interest:**

255 The authors declare no conflict of interest.

256

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Table 1. Comparison of gene content in the *Armadillidium vulgare* and *Armadillidium nasatum* genomes.

	Genes	Genes common to both species ^a	Species-specific genes ^b	Single-copy species-specific genes
<i>Armadillidium vulgare</i>				
Total nb proteins	19051	10815	1898	6338
Nb proteins of unknown function	8588	1388	871	2133
% proteins of unknown function	45.08%	12.83%	45.89%	33.65%
<i>Armadillidium nasatum</i>				
Total nb proteins	14636	9429	1037	4170
Nb proteins of unknown function	5353	2645	612	2397
% proteins of unknown function	36.57%	28.05%	59.02%	57.48%

^a clustered in 8,036 orthogroups

^b clustered in 1,104 orthogroups

Table 2. Comparison of repeat content in the *Armadillidium vulgare* and *Armadillidium nasatum* genomes.

	<i>Armadillidium vulgare</i> (a)		<i>Armadillidium nasatum</i> (b)		Genome size difference (a-b)	
	Count	Cumulative length (bp)	Count	Cumulative length (bp)	Cumulative length (bp)	% cumulative length
Genome	n/a	1725108002	n/a	1223175971	501932031	n/a
All TEs	2132032	854113876	1544121	484727234	369386642	73.59%
DNA transposons	986943	279615254	750157	157019308	122595946	24.42%
LINEs	796433	417726622	532873	247894416	169832206	33.84%
SINEs	37622	5933232	32834	4978843	954389	0.19%
LTRs	246423	135610359	179870	63190568	72419791	14.43%
Retroposons	64611	15228409	48387	11644099	3584310	0.71%
Tandem repeats	2 536 308	303620084	2047530	285430073	18190011	3.62%
Other repeats	227683	17582771	217427	20436134	-2853363	-0.57%

Table 3. Comparison of expressed copies of transposable elements in the *Armadillidium vulgare* and *Armadillidium nasatum* genomes.

	<i>Armadillidium vulgare</i>		<i>Armadillidium nasatum</i>	
	Count	%	Count	%
LINEs	2822	53.08%	657	52.18%
SINEs	6	0.11%	0	0%
LTRs	779	14.65%	258	20.49%
DNA transposons	1374	25.84%	318	25.26%
Retroposons	314	5.91%	11	0.87%
Others	22	0.41%	15	1.19%
All transposable elements	5317	100%	1259	100%

438 **Figure 1.** Venn diagram of the assignment of orthologous genes between *Armadillidium*
439 *vulgare* (red) and *Armadillidium nasatum* (blue). Proteins common to both genomes (9,429
440 for *A. nasatum* and 10,815 for *A. vulgare*) correspond to 8,036 orthogroups.

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Figure 2. Content of the *Armadillidium vulgare* (A) and *Armadillidium nasatum* (B) genomes.
 (C) Comparison of the cumulative length (in megabases) of various genomic categories in *A. vulgare* and *A. nasatum*.

Figure 3. Frequency distribution of transposable element families according to the divergence of individual copies to their respective family consensus sequences. Transposable elements identified in *Armadillidium vulgare* are shown in the upper part of the figure and those identified in *Armadillidium nasatum* in the lower part. The red arrow indicates the estimated genetic distance between *A. vulgare* and *A. nasatum*.

Figure 4. Comparison of the most expressed transposable element families in the *Armadillidium vulgare* (top) and *Armadillidium nasatum* (bottom) genomes. The cumulative expression (in reads per kilobase of exon model per million mapped) of the 15 most expressed families are shown. Error bars indicate standard deviations.