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Citation

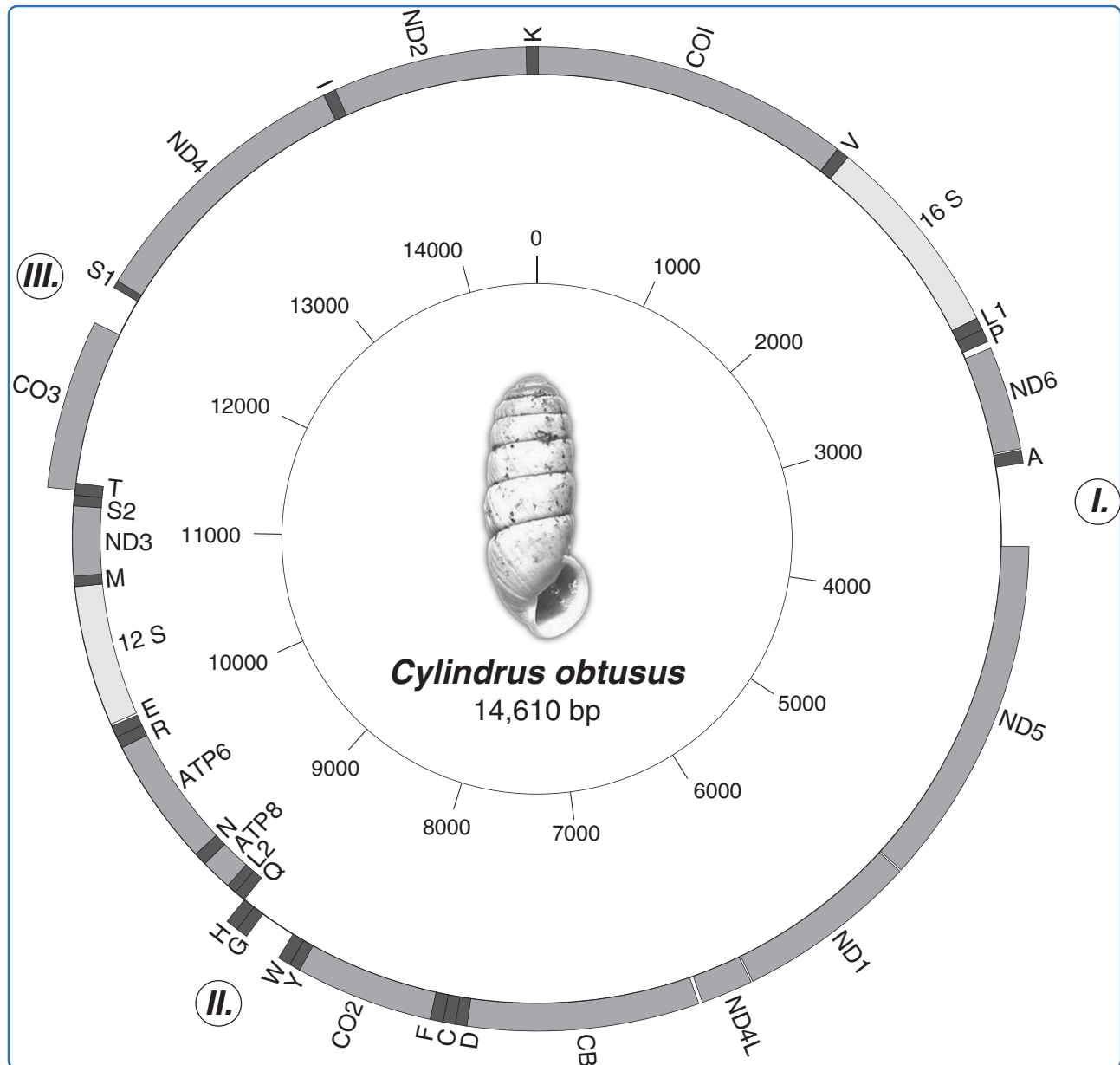
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Groenenberg *et al.*

METHODOLOGY ARTICLE

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The complete mitogenome of *Cylindrus obtusus* (Helicidae, Ariantinae) using Illumina next generation sequencing

Dick SJ Groenenberg^{1*}, Walter Pirovano², Edmund Gittenberger^{1,3} and Menno Schilthuizen³

Abstract

Background: This study describes how the complete mitogenome of a terrestrial snail, *Cylindrus obtusus* (Draparnaud, 1805) was sequenced without PCRs from a collection specimen that had been in 70% ethanol for 8 years. The mitogenome was obtained with Illumina GALLx shot gun sequencing. Although the used specimen was collected relatively recently and kept in a DNA-friendly preservative (not formalin as frequently used with old museum specimens), we believe that the exclusion of PCRs as facilitated by NGS (Next Generation Sequencing) removes a great obstacle in DNA sequencing of collection specimens. A brief comparison is made between our Illumina GALLx approach and a similar study that made use of the Roche 454-FLX platform.

Results: The mtDNA sequence of *C. obtusus* is 14,610 bases in length (about 0.5 kb larger than other stylommatophoran mitogenomes reported hitherto) and contains the 37 genes (13 protein coding genes, two rRNAs and 22 tRNAs) typical for metazoans. Except for a swap between the position of tRNA-Pro and tRNA-Ala, the gene arrangement of *C. obtusus* is identical to that reported for *Cepaea nemoralis*. The 'aberrant' rearrangement of tRNA-Thr and *COIII* compared to that of other Sigmurethra (and the majority of gastropods), is not unique for *C. nemoralis* (subfamily Helicinae), but is also shown to occur in *C. obtusus* (subfamily Ariantinae) and might be a synapomorphy for the family Helicidae.

Conclusions: Natural history collections potentially harbor a wealth of information for the field of evolutionary genetics, but it can be difficult to amplify DNA from such specimens (due to DNA degradation for instance). Because NGS techniques do not rely on primer-directed amplification (PCR) and allow DNA to be fragmented (DNA gets sheared during library preparation), NGS could be a valuable tool for retrieving DNA sequence data from such specimens. A comparison between Illumina GALLx and the Roche 454 platform suggests that the former might be more suited for *de novo* sequencing of mitogenomes.

Background

Although NGS techniques advanced rapidly over the last years and sequencing of entire mitochondrial genomes (mitogenomes) has consequently become more common [1-3], knowledge about stylommatophoran ('terrestrial snails') mitogenomes seems to have advanced at a slower pace. For over two decades, the complete mitogenomes of only two stylommatophorans, *Cepaea nemoralis* [4] and *Albinaria caerulea* [5], had been known. Of a third species, *Euhadra herklotsi*, most of the mitogenome has

been covered, so comparisons of mitochondrial gene arrangements could be made [6-8], but a complete sequence for that mitogenome is still missing [6,9,10]. Sequencing mitogenomes has been quite cumbersome because enrichment of the mitochondrial fraction (e.g. physical isolation of mitochondria, cloning of large mitochondrial fragments, long range, simplex or multiplex PCR) was quite laborious [1-3,8,11-15]. Moreover, the throughput of traditional (Sanger) sequencing is limited and sequencing of larger (> 1 kb) fragments is often delayed by the development of internal primers ('primer walking'). With NGS technologies, primer-directed amplification is no longer necessary and genome

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sequencing, mitogenomes in particular because of the small size and high-copy-number, has become fast and easy.

Nearly all traditional (Sanger) [1-3,8,11-15] and still some NGS [16] approaches for sequencing mitogenomes rely on long range PCR amplification. Due to the often degraded state of the DNA, this by and large excludes the use of specimens from natural history collections. An alternative is the hybridization capture approach [17], but this requires *a priori* sequence knowledge in order to design probes. Although enrichment of the mitochondrial fraction by long range PCR will increase the chance of obtaining a complete mitogenome (and facilitate the use of multiple specimens if sequence tags are exploited; [18]), it is not essential to NGS (e.g. [19-21]). In fact, the first step in NGS library preparation is fragmentation of the DNA. Consequently, DNA sequence data might be obtained from specimens of natural history collections with NGS, where PCR-based approaches fail. Whether NGS will allow, e.g. the recovery of complete mitogenomes from collection specimens, will depend on various parameters such as: the extent of DNA degradation, the ratio of nuclear to extrachromosomal DNA (which depends on the size of the genomes as well as on the type of tissue selected) and the number and length of the obtained sequences (dependent on the selected NGS platform). To the best of our knowledge, mitogenomes from NGS studies are thus far obtained either with the use of a long PCR enrichment procedure [16] prior to the NGS run, or with traditional Sanger sequencing after the run (to close the gaps remaining after assembly of the mitogenome, or to get an acceptable coverage) [19,20,22]. Since each of these approaches relies on PCR, both can be impracticable for (fragmented) DNA retrieved from museum specimens. With this study we wanted to test whether it would be feasible (using the Illumina GAIIx platform) to obtain a complete mitogenome, without PCR, from a single museum specimen.

We selected *C. obtusus* because it is an interesting species from both a morphological and a biogeographic point of view. *C. obtusus* is endemic to the Austrian Alps where it can be found in calcareous areas [23] at altitudes nearly always above 1,600 m. It has a disjunct distribution, probably mirroring the small insular areas ('nunataks'; [24]) in which it survived the last glacial maximum (LGM). Except for some Late Pleistocene specimens [25] there is no fossil record for *C. obtusus*. *Cylindrus* constitutes a monotypic genus. Within the helicid subfamily Ariantinae it is aberrant by being the only species with a cylindrical shell (Figure 1); other members of this speciose subfamily have broadly depressed (e.g. *Campylaea*, *Helicigona* and *Chilostoma*) or globular (*Arianta arbustorum*) shells (Figure 2 in [26]).

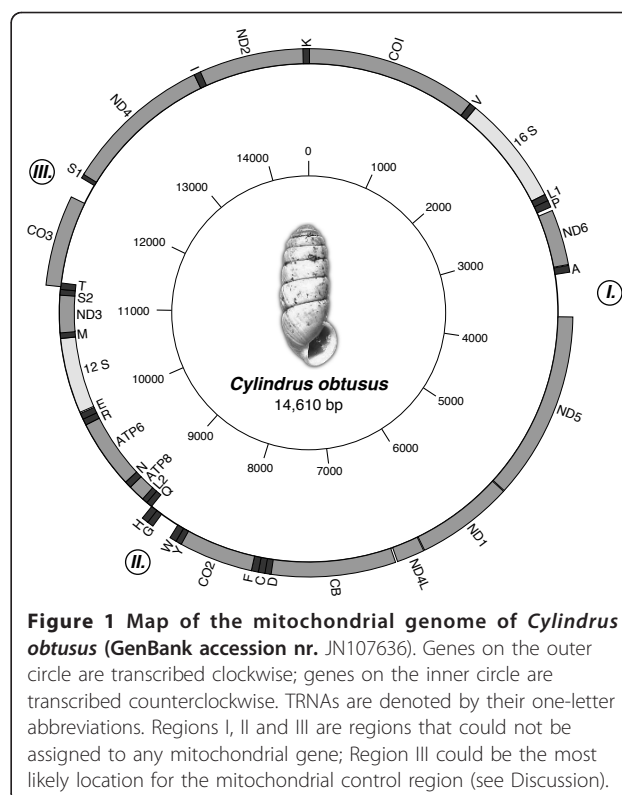


Figure 1 Map of the mitochondrial genome of *Cylindrus obtusus* (GenBank accession nr. JN107636). Genes on the outer circle are transcribed clockwise; genes on the inner circle are transcribed counterclockwise. TRNAs are denoted by their one-letter abbreviations. Regions I, II and III are regions that could not be assigned to any mitochondrial gene; Region III could be the most likely location for the mitochondrial control region (see Discussion).

This study shows that NGS can aid in the retrieval of sequence data (here a complete mitogenome) without using PCRs. Due to DNA degradation, PCRs are often a bottleneck for museum specimens. Based on our results for a specimen that has been in 70% ethanol for 8 years, we plea that NGS could be a promising technique for obtaining sequence data from museum specimens. We report the third complete mitogenome for a species of terrestrial snail ever published and compare our Illumina GAIIx strategy for sequencing mitogenomes with a similar study [20] in which the 454 platform of Roche was deployed.

Methods

Collection and preservation

Specimens of *C. obtusus* were collected by J. Gould in 2001 in Großer Buchstein (3.5 km NW of Gstatteboden), Ennstaler Alpen (Austria; 47°37' N 14°36' E) at an elevation of 2,200 m. After collection the specimens were drowned in water and subsequently placed in ethanol 70%. Finally they were stored in the molluscan wet collection of NCB Naturalis under collection number RMNH. MOL.144846.

DNA extraction and quality assessment

DNA was extracted from a single specimen with a DNeasy Blood and Tissue kit (Qiagen). Apart from using a total of 40 µl of Proteinase K and overnight

lysis, the manufacturer's instructions were followed. The DNA concentration of the extract was measured on a Nanodrop 1000 spectrophotometer (Thermo Scientific) and checked on an agarose gel.

Confirmation of NGS output

Because only a small number of *C. obtusus* (microsatellite) sequences were present in GenBank [27], we tried to sequence *COI* and *CytB* which would allow the identification of mitochondrial contig sequences (expected GAIx output). To do so, the following primers were selected: L1490 & H2198 for *COI* [28] and UCYTB151F & UCYTB270R for *CytB* [29]. PCRs were performed in 25 µl volumes using 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 mM of each primer and 0.25 µl (1.25 units) of Taq DNA polymerase (Qiagen). A thermoprofile of 3 min. at 94°C, followed by 40 cycles of 15 sec. at 94°C, 30 sec. at 50°C and 40 sec. at 72°C, and a final extension of 5 min. at 72°C was used for both markers. The PCR products were sent to Macrogen Europe (Amsterdam) where they were purified with a Montage purification kit (Millipore) and sequenced in both directions (using the same primers that were used for PCR) on an ABI3730XL. Contigs of forward and reverse sequences were assembled with Sequencher v. 4.10.1.

Assessing limitations of the DNA extract: long range PCR

Although the aim of this study is to assess the possibility of sequencing a complete mitogenome without PCRs from a collection specimen, the underlying assumption is that enrichment of the target sequence(s) by primer-directed amplification will be difficult or impossible for these kinds of object. To test this assumption, we tried to enrich the mitochondrial fraction of the obtained DNA extract by means of long range PCR. To increase the chance of successfully amplifying the complete mitochondrion, two *Cylindrus* specific primer sets (A and B) were designed that each amplified (roughly) half of the mitochondrion. These two primer-sets were tested with the "Expand Long Template PCR System" of Roche (Cat. No. 11 681 834 001), following the manufacturer's protocol. Primer-sets A and B were designed with Primer3 [30] and face outward of the obtained *COI* and *CytB* sequences:

A-Cobt-COI- 5'-TTACAACATTTTTTAATATGCGTTCTCCT-3' &

A-Cobt-CB- 5'-CGACGAGAAATAAACATTTAA-CATAACTA-3' and

B-Cobt-CB- 5'-TACCTTTTGTGATTAGTGTTT TTGTGTTAT-3' &

B-Cobt-COI- 5'-TATTATTTATCCGGGGAAACCT-TATATC-3'

We assumed that the orientation of *COI* and *CytB* would be identical to that of *C. nemoralis*.

GAIx library preparation

For DNA extracts from fresh tissues, the first step in library preparation is fragmentation of (genomic or PCR amplified) DNA. For extracts from museum specimens, DNA can already be expected to be fragmented, which would make this step unnecessary or even detrimental ([17] and references therein). The extent of DNA degradation will depend heavily on the preservation history. Based on the quality assessment of our DNA extract and the adverse effect that improperly fragmented DNA has on GAIx runs, we decided to follow a general library preparation procedure, for which we used the NEBNext™ DNA Sample Prep Kit (E6000-L, New England BioLabs). The DNA extract was randomly sheared with a nebulizer (K7025-05, Invitrogen) for 6 min. at 2.4 bar (35 psi) to obtain fragments in the range of 200-600 nucleotides. Fragments with a length of ca. 300 bp (insert-length without adaptors approx. 200 nucleotides) were extracted from an MS8-agarose gel (Talron Biotech. L.T.D.) with a Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA, USA). For all subsequent steps the NEBNext™ DNA Sample Prep Kit protocol was followed.

GAIx run and data analysis

The prepared library was run at BaseClear B.V. (The Netherlands) on a single lane of a GAIx flow cell. CLC Genomics Workbench version 4.0 (CLC Bio, Cambridge, MA, USA) was used to filter the output and for *de novo* assembly.

Annotation of the mitogenome

The contig sequence was annotated, based on similarity with available sequences in GenBank (Blast searches; <http://www.ncbi.nlm.nih.gov:80/BLAST/>), using pairwise alignments with, in particular, *C. nemoralis* and with the organellar genome annotation program DOGMA [31]. In order for DOGMA to detect all potential tRNAs (22 expected) we ultimately set the COVE-score to zero, but even then still some were missing. Therefore, the programs tRNAscan [32] and ARWEN [33] were used as well.

Results

DNA extraction

The DNA extract for the selected *C. obtusus* specimen had a yield 28 µg (200 µl buffer AE; concentration 139.8 ng/µl) and degradation as judged on the agarose gel was limited, considering the age of the specimen.

PCR assessment

The PCRs for the 396 bp and 705 bp (including primer sequences) fragments of *CytB* and *COI*, respectively, worked on the first attempt and were directly sequenced.

We failed to make the long PCRs work; with our *C. obtusus* specific primers (see Methods: Assessing limitations of the DNA extract), nor with any combination of the “universal” primers that successfully amplified the shorter *COI* and *CytB* sequences (L1490 & UCYTB270R; UCYTB151F & H2198).

Illumina GAIIX run

The GAIIX run resulted in 34,174,164 reads with an average read length of 50 nucleotides. Of these 685,537 reads were overlapping and used for a *de novo* assembly (CLC Bio version 4.0). This resulted in 740 contigs with a total length of 478,878 bp. The largest contig had a length of 14,610 nucleotides and an average coverage of 26.65 ×. The latter contig was identified as the mitogenome of *C. obtusus* based on the expected length (roughly 14 kb), the presence of the *COI* and *CytB* sequences (see Methods: Confirmation of NGS output) and on similarity with mitochondrial sequences from other styliomatophorans as resulting from Blast searches.

Initial assignment of PCGs and rRNAs

Twelve of the expected 13 protein-coding genes (PCGs), as well as both of the ribosomal RNAs (rRNAs) were recognized by DOGMA [31]; *ATP8* had to be located based on a pairwise alignment with *C. nemoralis* and *A. caerulea*. Although the gene arrangement (of the PCGs and rRNAs) as assigned by DOGMA seemed correct (compared to the gene arrangement for *C. nemoralis*), the program had difficulties determining the gene boundaries (most likely due to the absence of similar sequences on GenBank). Since we lack data from peptide sequencing for any of the PCGs, the putative gene boundaries (Table 1) were determined based on pairwise alignments with the amino acid sequences of *C. nemoralis*, *A. caerulea* and *E. herklotsi*. Nine of the PCGs start with a common initiation codon (ATA 5×; ATG 4×); the other four start with less common (but not unique for invertebrates) initiation codons (TTG 2×; ATC 1× and GTG 1×). For four PCGs (unrelated to the four just mentioned) we had to infer that they ended with a truncated termination codon (that is, the stop codon is most likely generated by posttranscriptional polyadenylation; [5,34]).

Annotation of rRNAs

The conserved regions at the beginning and end of both rRNAs (described in Figure 4 in [5]) were found in the contig sequence of *C. obtusus* as well. However, for both *A. caerulea* and *C. nemoralis*, the annotation of *rrnS* (12S) and *rrnL* (16S) extends beyond these conserved regions. Even though the exact gene boundaries of these ribosomal genes need to be confirmed by transcript mapping, the sequence data for *A. caerulea* and *C.*

nemoralis show little to no space between the rRNAs and the surrounding tRNAs. Consequently, we based the putative boundaries of 12S and 16S (Table 1) on alignments with sequences of the just mentioned species for those genes and the position of the surrounding tRNAs (described in the next paragraph).

Annotation of tRNAs

Because of the low COVE-score, half of the tRNAs assigned by DOGMA (17 out of 34) were false positives, but even with these relaxed settings tRNA-P(Pro), -G (Gly), -S₂ (Ser), -I(Ile) and -K(Lys) were missed. None of the missing tRNAs could be detected with tRNAscan. Using the least restrictive parameters, tRNAscan yielded no false positives, but merely detected four tRNAs (L₁ (Leu), -N(Asn), -M(Met) and -T(Thr)) that were already assigned by DOGMA. Of the three programs tested, only Arwen found 20 of the 22 tRNAs at the cost of just one false positive. When the output of ARWEN and DOGMA was combined, all tRNAs except tRNA-G(Gly) were assigned. Apart from a swap between tRNA-P and tRNA-A(Ala), the gene order for *C. obtusus* is identical to that of *C. nemoralis*. Alignments that included sequences of both *A. caerulea* and *C. nemoralis* [6,7] showed that tRNA-G is located between tRNA-W(Trp) and tRNA-H(His). In the annotated mitochondrion of *C. obtusus* (Figure 1), there is indeed an unassigned region between tRNA-W and tRNA-H. An alignment with sequences of *C. obtusus*, *A. caerulea* and *C. nemoralis* showed a conserved sequence, TACCTTCCAAG (8797-8809) within this non-annotated region of *C. obtusus*, which represents the anticodon loop and part of the anticodon stem of tRNA-G. Consequently, this alignment was used to infer the approximate location of tRNA-G, even though the secondary structure for this tRNA could not be predicted. Additionally, the location of each tRNA was confirmed by the presence of the anti-codon. A map of the mitogenome of *C. obtusus* is depicted in Figure 1. A summary of the mitochondrial genome content is given in Table 1. The corresponding annotated sequence was deposited in Genbank under accession number JN107636.

Discussion

At present, NGS techniques are being used increasingly in mitogenomic studies. One of the commonly used platforms is Roche 454 [16,19,20,22]. The ability to generate longer reads (which facilitates *de novo* assembly) and the reduction in sequence costs as compared to Illumina GAIIX, undoubtedly has added to the popularity of this platform. An overview of the costs and read lengths of these (and other) NGS platforms is given in Glen et al. [35]. When sequencing mitogenomes from museum specimens [21,36] the ability to sequence

Table 1 Summary of the mitochondrial genome content of *C.obtusius*

Name	One letter code	Position		Size	Strand	Nr. of amino acids	Anti-codon	Inferred initiation codon	Inferred termination codon
		From	To						
<i>COI</i>		1	1527	1527	H	508		TTG	TAA
<i>tRNA-Val</i>	V	1529	1589	61	H		TAC		
<i>16 S</i>		1590	2572	983	H				
<i>tRNA-Leu</i>	L1	2573	2635	63	H		TAG		
<i>tRNA-Pro</i>	P	2632	2694	63	H		TGG		
<i>ND6</i>		2727	3224	498	H	165		ATA	TAG
<i>tRNA-Ala</i>	A	3232	3293	62	H		TGC		
Region I		3294	3688	395					
<i>ND5</i>		3689	5368	1680	H	559		TTG	TAG
<i>ND1</i>		5377	6249	873	H	290		ATA	TAG
<i>ND4L</i>		6258	6507	250	H	83		ATA	T
<i>CB</i>		6526	7651	1126	H	375		ATG	T
<i>tRNA-Asp</i>	D	7637	7696	60	H		GTC		
<i>tRNA-Cys</i>	C	7692	7752	61	H		GCA		
<i>tRNA-Phe</i>	F	7752	7814	63	H		GAA		
<i>CO2</i>		7814	8500	687	H	228		ATG	TAG
<i>tRNA-Tyr</i>	Y	8475	8528	54	H		GTA		
<i>tRNA-Trp</i>	W	8529	8590	62	H		TCA		
Region II		8591	8771	181					
<i>tRNA-Gly</i>	G	8772	8832	61	H		TCC		
<i>tRNA-His</i>	H	8828	8889	62	H		GTG		
<i>tRNA-Gln</i>	Q	8891	8949	59	L		TTG		
<i>tRNA-Leu</i>	L2	8947	9004	58	L		TAA		
<i>ATP8</i>		8999	9157	159	L	52		GTG	TAG
<i>tRNA-Asn</i>	N	9158	9222	65	L		GTT		
<i>ATP6</i>		9222	9873	652	L	216		ATG	T
<i>tRNA-Arg</i>	R	9874	9935	62	L		TCG		
<i>tRNA-Glu</i>	E	9933	9992	60	L		TTC		
<i>12 S</i>		10004	10717	714	L				
<i>tRNA-Met</i>	M	10718	10779	62	L		CAT		
<i>ND3</i>		10770	11126	357	L	118		ATA	TAA
<i>tRNA-Ser</i>	S2	11123	11177	55	L		TGA		
<i>tRNA-Thr</i>	T	11177	11237	61	L		TGT		
<i>CO3</i>		11202	12014	813	H	270		ATG	TAA
Region III		12015	12203	189					
<i>tRNA-Ser</i>	S1	12204	12257	54	H		GCT		
<i>ND4</i>		12240	13568	1329	H	442		ATC	TAA
<i>tRNA-Ile</i>	I	13569	13631	63	H		GAT		
<i>ND2</i>		13628	14606	979	H	326		ATA	T
<i>tRNA-Lys</i>	K	14559	7	59	H		TTT		

longer DNA fragments likely is of no advantage and such studies generally rely on platforms that are optimized for short fragments, such as Illumina GAIIx. Despite the momentum that NGS has provided for ancient DNA research [17], the number of mitogenomic studies that actually use these new techniques to exploit natural history collections still is rather limited. This

paper provides an example of how NGS technology can be used to retrieve genetic information from a museum specimen. We show that it is feasible to sequence a complete mitogenome, without PCR, from a snail that has been in 70% ethanol for eight years.

Thus far, to the best of our knowledge, only [20] made use of an NGS platform to sequence the complete

mitogenome of another gastropod. In that study, a similar approach (no prior enrichment of the mitochondrial fraction) was taken, albeit with freshly collected specimens and a different NGS platform (Roche 454-FLX). Despite the commonalities, there were some noteworthy differences between both studies as well. Firstly, [20] used 13 specimens to obtain a complete mitogenome, whereas our results were obtained from a single specimen. The rationale behind using this many specimens is not given by the latter authors; perhaps they wished to account for intraspecific heterogeneity between the selected populations. It had nothing to do with the 'sensitivity' of the different platforms; the amount of DNA that Feldmeyer *et al.* [20] used for their sample preparation (6 µg), was roughly similar to what was used in this study (5 µg). The ability to obtain a complete mitogenomic sequence from a single specimen obviously is an advantage. Secondly, in contrast to our GAIIX run, the 454 run did not cover the complete mitogenome, requiring the design of additional primers and Sanger sequencing to close the three gaps that were left over after assembly. This is also observed in other mitogenomic studies in which the 454 platform was used without prior enrichment of the mitochondrial fraction [19,22]. After filtering, the 454 run resulted in 114 reads with an average length of 318 nt [20] that could be assigned to the mitochondrial genome, compared to 7,808 reads (out of 34,174,164) with an average length of 50 nt obtained with GAIIX. Thus the 454 run resulted in 36,252 mitogenomic nucleotides, whereas our GAIIX run yielded 390,400. The maximum coverage obtained with 454 and GAIIX is 2.6× and 26.7× for *R. balthica* and *C. obtusus* for mitogenome sizes of 13,993 bp and 14,610 bp, respectively. Generally longer reads as obtained with the 454 platform facilitate *de novo* assembly and might be preferred when little *a priori* sequence information is available ([37] and references therein). When reconstructing the just mentioned mitogenomes the sheer number of short reads generated by the GAIIX platform outcompeted the smaller number of longer reads as obtained with 454 sequencing.

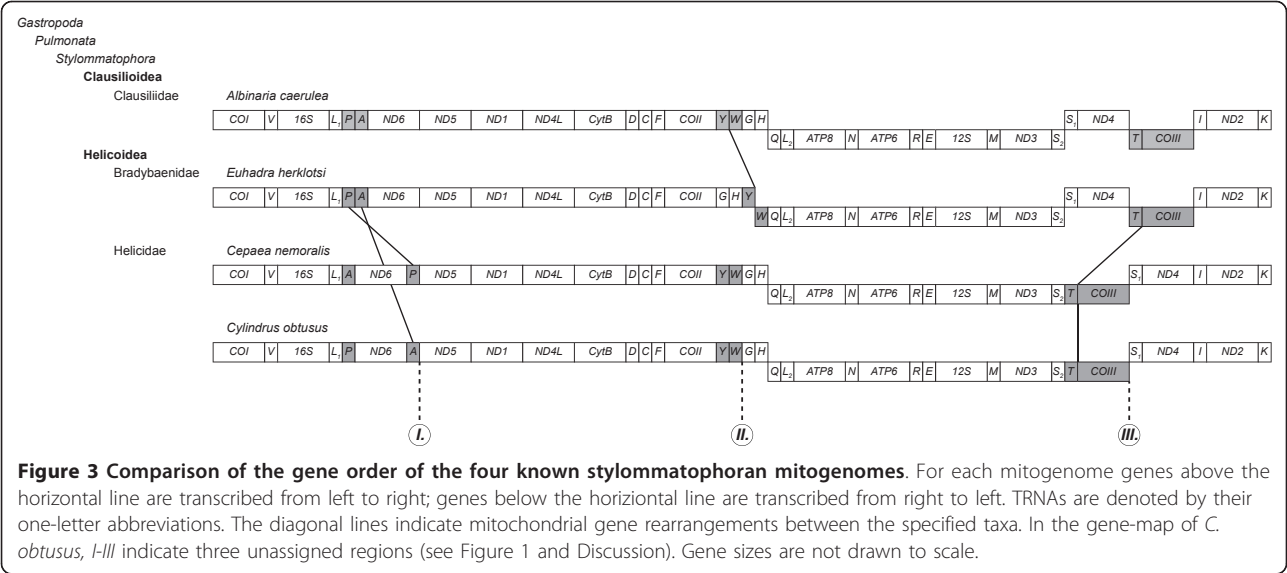
Identification of tRNAs in nematodes [38] and stylommatophorans [4-6] can be difficult because the standard cloverleaf secondary structure may not be present (T or D arms can be lacking; see tRNA-H, -S₁ and -S₂ in Figure 2) and pulmonate tRNAs can undergo post-transcriptional processing [7,39]. In a number of instances different tRNAs were predicted in the same approximate nucleotide region, depending on the algorithm used. The hypothetical tRNAs differed a few nucleotides in length, or were shifted a few bases, or both, causing a slight shift in the anticodon region. Despite the fact that only one of the tRNAs will be real, both algorithms correctly point to (roughly) the same nucleotide region for the placement of a tRNA.

In other instances, the different algorithms predicted tRNAs on exactly the same position but on opposite strands, causing the tRNAs to be the reverse-complement of each other [40]. Examples of the latter within *C. obtusus* are predictions by DOGMA of tRNA-W and tRNA-C on position 3632-3694 and 3232-3293, for which ARWEN predicted tRNA-P and tRNA-A, respectively. For both tRNAs, only those predicted by ARWEN were in agreement with existing annotations for related species in GenBank.

Although gene rearrangements are common in the mitogenomes of molluscs [7,8] and gastropods in particular [2,3,41], little is known about the mitochondrial gene organisation of terrestrial snails. The mt gene arrangements depicted by Yamazaki *et al.* [6] and Boore *et al.* [8] show less similarity between *C. nemoralis* and *E. herklotsi* (both species belonging to the Helicoidea), than between each of them and *A. caerulea* (a species belonging to the Clausilioidea). Based on a three-taxon statement Yamazaki *et al.* [6] concluded that the rearrangement of the tRNAs between *COII* and *ATP8* represented a derived state in *E. herklotsi*. Similarly, they concluded that the positions of tRNA-P and the gene-region tRNA-T/*COIII* represented a derived state in *C. nemoralis*. By comparing these gene rearrangements with the gene order observed in *C. obtusus*, we can get some insight in the evolution of the mitochondrial gene order of the Helicoidea. Figure 3 gives an overview of the gene organisation of the four stylommatophoran mitogenomes currently known. Starting at *COI* the first observed rearrangement is the relocation of tRNA-P within *C. nemoralis*. Although the location of tRNA-P in *C. obtusus* is the same as that found within the other stylommatophorans, the gene region itself seems to be of high potential for rearrangements within the Helicidae. In *C. obtusus* we see that instead of tRNA-P, tRNA-A has been relocated between *ND6* and *ND5*. Thus within the Stylommatophora, the relocation of a tRNA from the L₁, A, P-region to between *ND6* and *ND5* seems to be a synapomorphy for the Helicidae. The second gene rearrangement (Figure 3) is found in *E. herklotsi* within a series of tRNAs located between *COII* and *ATP8*. Despite the fact that these tRNAs (Y, W, G, H, Q, L₂) have rearranged frequently during gastropod evolution [2,3], the gene order seems rather conserved within the Eupulmonata. The five eupulmonates currently listed in the genome database of NCBI and *C. obtusus* all show the order Y, W, G, H, Q, L₂. Therefore, we endorse the conclusion of Yamazaki *et al.* [6] that the arrangement of these tRNAs as observed in *E. herklotsi* can be considered a derived state (and possibly represents an apomorphy for the Bradybaenidae). Presence of the ancestral tRNA arrangement in both *C. obtusus* and *C. nemoralis*, suggests that that gene order has not changed in the Helicidae. The last gene rearrangement is the relocation of the



Most metazoan mitogenomes possess a single major non-coding region presumed to contain the signals for replication and transcription. This region is usually referred to as the control region [42]. In some groups of



invertebrates, such as gastropods [2,5] and spiders [43] the mitogenomes can be very compact, hardly leaving any non-coding regions of significant length. Although the mitogenome of *C. obtusus* (14,610 bp) is still compact compared to that of other Metazoa (approx. 15-24 kb; [44]), it is about half a kb larger than the mitogenomes of *C. nemoralis* (14,100 bp) and *A. caerulea* (14,130 bp). The schematic overview of this mitogenome (Figure 1) shows three unassigned regions (indicated as I, II and III) with a length of 394, 181 and 189 nt, respectively. When the mitochondrial gene order of *C. obtusus* is compared with that of other stylommatophorans currently known (Figure 3), it becomes clear that region I and III coincide with the transposition of tRNA-A and the region tRNA-T/COIII. As for region II, no gene rearrangement was observed in *C. obtusus* and neither *C. nemoralis* nor *A. caerulea* have any unassigned sequence between tRNA-W and tRNA-G. But then *E. herklotsi* does show a gene rearrangement in that region (Figure 3). In stylommatophorans (or all gastropods for that matter) the location of the mitochondrial control region is still a subject of debate. Given the absence of region II in other stylommatophorans, we believe that of the three non-coding regions, region II is the least likely location for the control region. As for region I, Grande et al. [9] suggested that the region between ND6 and ND5 might contain recognition signals for transcription in the nudibranch *Roboastra europaea*. Except for *Onchidella celtica*, which, like *C. obtusus*, has its longest non-coding sequence here [2], most of the heterobranch gastropods sequenced thus far show very little unassigned sequence between ND6 and ND5. Therefore we assume that region I is not the most likely location for the control region either. Within the

heterobranch gastropods the region between COIII and tRNA-I is most often cited as the potential location for the control region [2,3,9,45]. Because of the shown transposition of tRNA-T/COIII within *C. nemoralis* and *C. obtusus* (Figure 3), this region was transposed as well. Thus for those species (and likely all Helicidae), the potential control region (the region adjacent to COIII) is not located between COIII and tRNA-I, but between COIII and tRNA-S₁. For *A. caerulea* and *Pupa strigosa*, the presence of 10 nt (or 20 nt if the 5 nt overlap with tRNA-I is included) and 25 nt palindromes, respectively [5,45], has been implicated to function as a bidirectional promotor for this putative control region. Within *C. obtusus*, *C. nemoralis* and *E. herklotsi*, the palindromes found in this region were never larger than six nucleotides. We were unable to confidently align the putative control region for the four mentioned stylommatophorans. The length of this region was longer in the Helicidae (*C. obtusus* = 189 nt; *C. nemoralis* = 158 nt) than in the other two stylommatophorans (*A. caerulea* = 42 nt; *E. herklotsi* = 43 nt), which is most certainly related to the transposition of tRNA-T/COIII. Besides the apparent consistency of the presence of a non-coding region adjacent to COIII, there still is little to go on for recognition of the control region in heterobranch gastropods. Given the limited number of available stylommatophoran (and even eupulmonate) mitogenomes (GenBank), we consider more extensive genomic comparisons (such as [46]) to be premature, based on these data.

Although this study illustrates the potential of NGS to obtain genetic information from museum specimens, there are some caveats that need to be addressed. As for the results of the long range PCR, we did not have a recently collected specimen of *C. obtusus* at our disposal.

Therefore, we have not shown that the failure of the long range PCR was caused by the fact that we used an 'aged' collection specimen. Otherwise, no products would have been obtained with the long range PCRs either, based, as they were, on the universal primers, which were shown to work with *C. obtusus*. The fact that an NGS approach worked well in this study, also does not imply that NGS approaches will always be fruitful when applied to collection specimens. A vast number of parameters such as the effect of fixatives, time (the "age" of specimens) and preservation history were not assessed in this study. Also annotation of a mitogenome largely based on similarity with available sequences in GenBank (as opposed to transcript mapping or peptide sequencing) is hazardous. Closely related species might not be present and existing annotations are not guaranteed to be flawless [47,48]. Another hypothetical problem is that due to the relative short length of the obtained sequences (50 nucleotides on average) repeats within the mitogenome could be missed. The mitogenomes of gastropods however are very compact, none of stylommatophorans sequenced thus far show such repeats and the length of the complete mt sequence is similar to that of other stylommatophorans. We therefore assume that the mitogenome of *C. obtusus* presented here is complete. Based on the sheer number of sequences generated with GAIIX and 454, we are convinced that without PCRs it will be more difficult to obtain a complete mitogenome with the latter platform (despite the longer reads). Even though our GAIIX approach is reasonably similar to the 454 approach described by Feldmeyer *et al.* [20], the comparison will not be conclusive as long as the total genome sizes of *C. obtusus* and *R. balthica* are unknown. The average genome size (<http://www.genomesize.com>) of available stylommatophorans (2.86 c or 2.79 GB) compared to basommatophorans (1.34 c or 1.31 GB) nevertheless suggest that it will be more difficult to obtain the mitogenome of *C. obtusus* than that of *R. balthica*. It is likely that the assembly of mitogenomes will benefit from the advances in NGS technologies (e.g. the Illumina HiSeq platform), as well as from the promising arrival of third generation sequencers (e.g. the PacBio RS platform).

Conclusions

On a par with previous studies [21,36], this study shows that NGS can aid in the retrieval of mitogenomes from museum specimens. Although sequencing of mitogenomes by means of NGS without an enrichment procedure is very inefficient (only 0.02% of the reads from our GAIIX run were used for assembly of the mitogenome), it eliminates the use of PCRs which is often a bottleneck for degraded DNA samples. Without prior enrichment of the mitochondrial fraction, the GAIIX platform (Illumina) might be better suited for *de novo* sequencing of

mitogenomes than the 454 platform (Roche). Besides being much faster than conventional sequencing (which generally results in 2× coverage), sequencing of mitogenomes by means of NGS also yields higher confidence estimates (on average 26× times coverage, in this study). Except for a swap between tRNA-P and tRNA-A, the mitochondrial gene arrangement of *C. obtusus* is identical to that of *C. nemoralis*. Within the Helicidae the region tRNA-L₁, P, A might be a hot spot for transposition of genes (in particular to the region between *ND6* and *ND5*). The location of tRNA-T/*COIII* between *ND3* and *ND4* (instead of between *ND4* and *ND2*) might be an apomorphy for the family Helicidae. We hope that the results of this study will aid to future studies on stylommatophoran evolution and the phylogeny of the subfamily Ariantinae in particular.

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Authors' contributions

DSJG conceived and designed the study, carried out the DNA extraction, performed the annotations and wrote the manuscript. WP carried out the mitogenome assembly and helped to draft the manuscript. EG and MS participated in the design of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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