Extensive gene rearrangements in the mitochondrial genomes of two egg parasitoids, *Trichogramma japonicum* and *Trichogramma ostriniae* (Hymenoptera: Chalcidoidea: Trichogrammatidae)

Long Chen¹, Peng-Yan Chen²,³, Xiao-Feng Xue¹, Hai-Qing Hua¹, Yuan-Xi Li¹, Fan Zhang² & Shu-Jun Wei²

Animal mitochondrial genomes usually exhibit conserved gene arrangement across major lineages, while those in the Hymenoptera are known to possess frequent rearrangements, as are those of several other orders of insects. Here, we sequenced two complete mitochondrial genomes of *Trichogramma japonicum* and *Trichogramma ostriniae* (Hymenoptera: Chalcidoidea: Trichogrammatidae). In total, 37 mitochondrial genes were identified in both species. The same gene arrangement pattern was found in the two species, with extensive gene rearrangement compared with the ancestral insect mitochondrial genome. Most tRNA genes and all protein-coding genes were encoded on the minority strand. In total, 15 tRNA genes and seven protein-coding genes were rearranged. The rearrangements of *cox1* and *nad2* as well as most tRNA genes were novel. Phylogenetic analysis based on nucleotide sequences of protein-coding genes and on gene arrangement patterns produced identical topologies that support the relationship of (Agaonidae + Pteromalidae) + Trichogrammatidae in Chalcidoidea. CREx analysis revealed eight rearrangement operations occurred from presumed ancestral gene order of Chalcidoidea to form the derived gene order of *Trichogramma*. Our study shows that gene rearrangement information in Chalcidoidea can potentially contribute to the phylogeny of Chalcidoidea when more mitochondrial genome sequences are available.

A typical animal mitochondrial genome is composed of 13 protein-coding, 22 tRNA and two rRNA genes, and a major non-coding sequence called the control region. The sequences and structural features of mitochondrial genomes, such as the secondary structure of RNA genes, gene content and gene arrangement, reflect differences in function and evolutionary pattern in different taxa. As an increasing number of mitochondrial genomes have been obtained, comparative feature analysis has become feasible among and within certain groups. Gene rearrangement is one of the most frequently investigated features in animal mitochondrial genomes. Comparative studies have shown that gene arrangements are usually conserved across major lineages but may be rearranged within some groups. In insects, gene rearrangement has been reported in most orders. Accelerated rates of gene rearrangement have been found particularly in species of hemipteroids (thrips, book lice, lice), Protura and Hymenoptera (wasps, ants and bees). It has been known that the gene order of mitochondrial genome contains phylogenetic signals since the seminal work of Sankoff, et al. and Boore, et al. However, no gene rearrangements are shared between insect orders. Examining gene rearrangement within lower taxonomic lineages of insects is expected to shed light on the evolution of these groups.

¹Department of Entomology, Nanjing Agricultural University, Nanjing, 210095, China. ²Institute of Plant and Environmental Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing, 100097, China. ³Department of Entomology, South China Agricultural University, Guangzhou, 510640, China. Correspondence and requests for materials should be addressed to Y.-X.L. (email: yxli@njau.edu.cn) or S.-J.W. (email: shujun268@163.com)
Ancestral pattern of insect mitochondrial gene arrangement

1. O M mtr2 W E L Y
2. c ox1 l2 g
3. K D 8 g66
4. tRNA cluster
5. T D 6

Hypothetical arrangement

Translocation of cox3-trnG

Inverse transposition of tmF

Figure 1. Ancestral arrangement of insect mitochondrial genes and types of gene rearrangement. The numbers 1 to 5 in circular indicate the five tRNA clusters. Transposition, inversion, inverse transposition were illustrated by comparing the ancestral pattern of insect mitochondrial gene arrangement and a hypothetical pattern.

Comparative studies may also contribute to understanding the forces that drive gene rearrangement. Gene rearrangements have been hypothesized to be correlated with parasitic life histories in the Hymenoptera and to some characteristics, such as body size and developmental time. Frequent gene rearrangements have been observed in apocritan Hymenoptera based on broad examinations of whole or partial mitochondrial genome sequences. Moreover, it has been reported that gene rearrangement was accelerated in the mitochondrial genomes of Apocrita. However, no tight association was found between an increased rate of mitochondrial gene rearrangement and the evolution of parasitism in an analysis of the characterization of 67 mitochondrial tRNA gene rearrangements in the Hymenoptera.

Gene rearrangement patterns in the Hymenoptera are usually complicated and variable compared with those in most other insect orders. Rearrangement of mitochondrial gene can be described by transposition, inversion, transposition and TDRL (tandem duplication random loss). Bernt et al. introduced a movement of TDRL to describe the duplication of multiple contiguous genes and the successive random loss of one of the two copies. In the Hymenoptera, rearrangements of tRNA genes usually occurred around the five gene clusters. Inversion of "tmH from the minority strand (heavy strand in mammal mitochondrial genomes) to the majority strand (light strand in mammal mitochondrial genomes)" has also occurred multiple times in the Braconidae (Hymenoptera). The rate of rearrangement of protein-coding genes is lower than that of tRNAs in the Hymenoptera. Rearrangement of protein-coding gene has been reported in limited species of Agaonidae (Chalcidoidea), Aulacidae (Evanioidea), Trigonalyidae (Trigonalyoidea), Pteromalidae (Chalcidoidea), Ichneumonidae (Ichneumonoidea), Braconidae (Ichneumonoidea) and Bethylidae (Chrysidoidae).

In the Chalcidoidea (Hymenoptera: Apocrita), unusually high rates of gene rearrangement, including not only tRNA genes but also protein-coding genes, have been found. Combined with the diverse lifestyles among species of this group, this high rearrangement rate provides suitable materials for examining the evolution of gene rearrangement. Presently, only a few complete or partial mitochondrial genomes are known from Chalcidoidea, including those from Megaphragma, Nasonia, and Philoloptritis. The Trichogrammatidae (Chalcidoidea) are small egg parasitoids with a short developmental duration and are one of the most important groups used in the biological control of insect pests. The parasitoids in this family can parasitize the eggs of about 10 orders. However, no complete mitochondrial genome has previously been sequenced from members of this family except for the mitochondrial genome sequence from Megaphragma. Increasing knowledge of the mitochondrial genomes of egg parasitoids will provide further insight into their higher-level phylogeny and the evolution of their life histories.

In the study, we sequenced two mitochondrial genomes from Trichogramma ostriniae and Trichogramma japonicum. We found novel and extensive gene rearrangements in both species compared with the ancestral insect mitochondrial genome. Phylogenetic relationships within the Chalcidoidea were reconstructed using mitochondrial genome sequences and gene arrangement patterns.

Results and Discussion

Genome structure. The complete mitochondrial genome of T. japonicum (GenBank accession number: KU577436) and T. ostriniae (GenBank accession number: KU577437) were determined, with lengths of 15,962 bp and 16,472 bp, respectively. The sizes were well within the range found in other completely sequenced hymenopteran insects (from 15,137 bp in Idris sp. to 19,339 bp in Cephus cinerascens) (Table S1). All typical animal mitochondrial genes and control regions were identified in both circular genomes (Table 1).

In the mitochondrial genome of T. japonicum, a total of 547 bp of intergenic nucleotides ranging from 1 to 81 bp were found in 17 locations. The longest intergenic spacer (81 bp) was found between atp8 and trnD. In the mitochondrial genome of T. ostriniae, there were 870 bp intergenic spacer sequence distributed among 19 locations with lengths from 1 to 171 bp. The longest intergenic spacer (171 bp) was located between trnF and trnI. Long intergenic spaces have been identified in other insect mitochondrial genomes and were considered as possible results of gene rearrangement.

SCIENTIFIC REPORTS | (2018) 8:7034 | DOI:10.1038/s41598-018-25338-3
Overlapping genes are very common in arthropod mitochondrial genomes. In the mitochondrial genome of *T. japonicum*, a total of 19 bp of overlapping nucleotides were detected with a length from 1 to 7 bp, while in that of *T. ostriniae* there were 18 bp shared nucleotides in total, also ranging from 1 to 7 bp. In both species, the overlapping regions were found in the same five locations, i.e., atp6-atp8, nad4-nad4l, trnT-trnP, trnS2-nad1 and trnV-trnR. The other 10 pairs of genes in *T. japonicum* and 13 pairs of genes in *T. ostriniae* were directly adjacent, without overlapping or intergenic nucleotides.

The sequences of both mitochondrial genomes are biased in nucleotide composition [(A + T)% > (G + C)%] (Table S2), which is common in mitochondrial genomes of suborder Apocrita (Hymenoptera). The parameters of AT skew and GC skew are frequently used to reveal the nucleotide-compositional behavior of mitochondrial genomes. In both species, the AT skews of the majority strand were positive, while GC skews were negative, which indicated that the two mitochondrial genomes contained more A than T and more C than G nucleotides (Table S2), as reported for most hymenopteran insects (Table S1).

| Gene    | Strand | Position | Size | INT | Start/stop codon | Position | Size | INE | Start/stop codon |
|---------|--------|----------|------|-----|------------------|----------|------|-----|------------------|
| trnW    | –      | 1–66     | 66   | 0   | –                | 1–67     | 67   | 0   | –                |
| nad2    | –      | 67–1078  | 1014 | 0   | ATA T            | 68–1080  | 1014 | 0   | ATA TA           |
| trnQ    | –      | 1079–1146| 68   | 17  |                  | 1081–1148| 68   | 63  |                  |
| trnT    | –      | 1164–1230| 67   | 41  |                  | 1212–1277| 66   | 62  |                  |
| cox1    | –      | 1272–2807| 1536 | 15  | ATG TAA          | 1340–2875| 1536 | 1   | ATG TAA          |
| trnE    | +      | 2823–2889| 67   | 25  |                  | 2877–2942| 66   | 2   |                  |
| trnF    | –      | 2915–2978| 64   | 6   |                  | 2945–3009| 66   | 7   |                  |
| trnI    | –      | 2985–3051| 67   | 3   |                  | 3181–3247| 67   | 0   |                  |
| trnS1   | –      | 3055–3113| 59   | 67  |                  | 3248–3307| 60   | 151 |                  |
| trnN    | –      | 3181–3246| 66   | 20  |                  | 3459–3524| 66   | 0   |                  |
| trnC    | –      | 3267–3335| 69   | 52  |                  | 3525–3592| 68   | 106 |                  |
| cox3    | –      | 3388–4179| 792  | 35  | ATG TAA          | 3699–4490| 792  | 24  | ATG TAA          |
| atp6    | –      | 4215–4889| 675  | –7  |                  | 4515–5189| 675  | –7  |                  |
| atp8    | –      | 4883–5050| 168  | 81  | ATT TAA          | 5183–5350| 168  | 72  | ATT TAA          |
| trnD    | –      | 5132–5197| 66   | 12  |                  | 5423–5488| 66   | 7   |                  |
| trnK    | +      | 5210–5279| 70   | 14  |                  | 5496–5565| 70   | 9   |                  |
| cox2    | –      | 5294–5974| 681  | 0   | ATT TAA          | 5575–6255| 681  | 0   | ATT TAA          |
| trnL2   | –      | 5975–6040| 66   | 31  |                  | 6256–6321| 66   | 29  |                  |
| nad5    | –      | 6072–7757| 1866 | 1   | ATA TAA          | 6351–8033| 1868 | 6   | ATT TAA          |
| trnH    | –      | 7759–7825| 67   | 21  |                  | 8040–8102| 63   | 30  |                  |
| nad4    | –      | 7847–9190| 1344 | –7  | ATG TAA          | 8133–9476| 1344 | –7  | ATG TAA          |
| nad4l   | –      | 9184–9471| 288  | 1   |                  | 9470–9757| 288  | 0   | TAG              |
| trnT    | +      | 9482–9546| 65   | –1  |                  | 9758–9821| 65   | –1  |                  |
| trnP    | –      | 9546–9611| 66   | 6   |                  | 9821–9885| 65   | 13  |                  |
| nad6    | –      | 9618–10196| 579 | 33  | ATT TAA          | 9899–10471| 573 | 2   | ATG TAA          |
| cob     | +      | 10230–11369| 1140 | 0   | ATT TAA          | 10474–11613| 1140 | 19  | ATG TAA          |
| trnS2   | +      | 11395–11458| 64  | –2  |                  | 11633–11696| 64  | –2  |                  |
| nad1    | –      | 11457–12392| 936 | 0   | ATT TAA          | 11695–12630| 936 | 0   | ATT TAA          |
| trnL1   | –      | 12393–12457| 65  | 0   |                  | 12631–12700| 70  | 0   |                  |
| trnL    | –      | 12458–13857| 1400| 0   |                  | 13701–14067| 1367 | 0   |                  |
| trnA    | –      | 13858–13922| 65  | 14  |                  | 14068–14131| 64  | 10  |                  |
| trnG    | –      | 13937–14001| 65  | 0   |                  | 14142–14208| 67  | 0   |                  |
| trnS    | –      | 14002–14791| 790| 0   |                  | 14209–14983| 775 | 0   |                  |
| trnV    | –      | 14792–14857| 66  | –2  |                  | 14984–15051| 68  | –1  |                  |
| trnK    | –      | 14856–14920| 65  | 18  |                  | 15051–15113| 63  | 103 |                  |
| nad3    | –      | 14939–15301| 363| 0   | ATA TAA          | 15217–15576| 360 | 0   | ATA TAA          |
| trnM    | –      | 15302–15369| 68  | 0   |                  | 15577–15642| 66  | 0   |                  |
| control region | | 15370–15962 | 593 | | | 15643–16472 | 830 | |

Table 1. Annotation of the *Trichogramma japonicum* and *Trichogramma ostriniae* mitochondrial genomes. + indicates the gene is coded on majority strand while – indicates the gene is coded on minority strand. INT indicates the intergenic nucleotides. Positive values indicate intergenic nucleotides and negative values indicate overlapping nucleotides between two adjacent genes.
Transfer RNA and ribosomal RNA genes. In total, 22 tRNA genes were interspersed throughout the Trichogramma mitochondrial genomes, of which four were coded on the majority strand while 18 were coded on the minority strand. The tRNA genes ranged from 59 bp (trnS1 in T. japonicum) to 70 bp (trnK in T. japonicum), well within the range observed in other insects (Table 1). All tRNA sequences can be folded into the canonical cloverleaf secondary structure, except for trnS1 which lacked the dihydrouridine (DHU) arm. A lack of the DHU arm in trnS1 was found in the mitochondrial genomes of most insects and other metazoans. Variations in the lengths of the variable loop, DHU and TVC arms result in the different sizes observed in the tRNA sequences. In total four mismatches (U-U in trnY, trnW, trnG and trnC) were found in T. japonicum and five (U-U in trnY, trnW, trnG, trnC and trnN) in T. ostriniae. Mismatches were located mostly in the DHU and anticodon stems (Figure S1).

As with other insect mitochondrial genome sequences, the large and small ribosomal RNA genes (rrnL and rrnS) were encoded by the minority strand in the same location (between trnL1-trnA and trnG-trnV). In T. japonicum, the length of the rrnS gene was 790 bp with an A+T content of 87.72%, while the rrnL gene was 1400 bp with an A+T content of 88.36%. In T. ostriniae the length of the rrnS gene was 775 bp with an A+T content of 88.52%, while the rrnL gene was 1367 bp with an A+T content of 88.00%.

Protein-coding genes and codon usage patterns. In both the T. japonicum and T. ostriniae mitochondrial genomes, 11 of 13 protein-coding genes were encoded by the minority strand, while two (nad6 and cob) were encoded by the majority strand. All homologous protein-coding genes from the two species had the same length, except for nad3, nad6 and nad5 (Table 1).

In the mitochondrial genome of T. japonicum, the total length of the protein-coding genes was 11,202 bp, accounting for 70.18% of the entire genome. The average A+T content of the 13 protein-coding genes was 83.08%, ranging from 76.04% (cox1) to 90.80% (nad2) for individual genes. In the mitochondrial genome of T. ostriniae, the total length of protein-coding genes was 11,190 bp, accounting for 67.93% of the entire genome. The average A+T content of the 13 protein-coding genes was 83.25%, ranging from 76.37% (cox1) to 91.30% (nad2) for individual genes (Table S2).

The predicted initiation codons are ATN, as in most other insect mitochondrial genomes. In some cases, a given gene may have different start codons in different species. There were seven genes (nad2, nad3, nad1, nad4L, nad5, cox2 and atp8) starting with ATG and five genes (cox1, cob, nad4, atp6 and cox3) starting with ATA in both genomes. In T. ostriniae, nad6 started with ATG, but in T. japonicum it started with ATA. All protein-coding genes terminated at the most common stop codon, TAA, in both genomes, except for nad4L in T. ostriniae, which stopped with TAG, and nad2, which stopped with T and TA in T. ostriniae and T. japonicum, respectively.

Codons with high A/T content were preferred in these two species, as in most insect mitochondrial genomes. In both species of this study, Ala, Gly, Leu, Pro, Arg, Ser, Thr and Val were the most frequently used amino acids, and UUA (Leu) had the highest relative synonymous codon usage (RSCU) (Table S3). All remaining codons with T were found in T. ostriniae.

Control region. Complete control regions were found in both species. The length of the control region was 593 bp in T. japonicum and 830 bp in T. ostriniae, which was well within the range reported in insects. The control region in both species was flanked by trnW and trnM with high A+T content (90.99% in T. japonicum and 89.03% in T. ostriniae).

The control region is believed to function in the initiation of replication and control of transcription of the mitochondrial genome. This region is usually characterized by five conserved elements as reported in some insect mitochondrial genomes. All of those elements could be identified in the mitochondrial genomes of Trichogramma, such as (1) a polyT stretch at the 5′ end of the control region; (2) a [TA(A)]n-like stretch following the polyT stretch; (3) a stem and loop structure (Figure S2); (4) a TATA motif and a G(A)nT motif flanking the stem and loop structure; and (5) a G + A-rich sequence downstream of the stem and loop structure. However, they were not in the typical orders and positions, as reported in some insect species.

Concerted evolution is common in the insect control region, most obviously in species with repeat units in their control regions such as termites but also in species with non-tandemly repeated control regions such as thrips. Repeat sequences were found in both species of Trichogramma, as have been reported in some other insects. In T. japonicum, three 21-bp tandem repeats of “AGCCCTAAAATCGGTTT” and two 41-bp tandem repeats of “ATTATAATTATTATATTATATAATATTATATAATATT” were found in the control region. In the three 21-bp tandem repeats, three mutations (“GCC” to “CTT”) in the first repeat region were present. The control region of T. ostriniae contained nine 21-bp tandem repeats with several mutations among repeat units (Figure S3). There was an 80-bp perfect repeat of TA in control region of T. ostriniae. The presence of repeat regions may inhibit DNA polymerase and could lead to the failure of sequencing in those regions.

Gene arrangement. In previously studied insect mitochondrial genomes, most rearranged genes were tRNA genes. In the Hymenoptera, numerous rearrangements of protein-coding genes have been identified in several groups. Compared with the putative ancestral pattern of the insect mitochondrial genome, dramatic gene rearrangements, not only in tRNA genes but also in protein-coding genes, were found in Trichogramma mitochondrial genomes. In total, 15 of 22 tRNA genes and seven of 13 protein-coding genes were rearranged in Trichogramma compared with the ancestral arrangement (Fig. 2). All genes in the mitochondrial genomes of the two Trichogramma species were encoded by the minority strand, rather than the majority strand, except for two protein-coding genes (cob and nad6) and four tRNA genes (trnE, trnK, trnL′ and trnS2).

Compared with the other sequenced mitochondrial genomes of Chalcidoidea, cox1 was inverted separately in Trichogramma, not together with the gene block of cox1-trnL2. The protein-coding gene nad2 did not change its relative position but changed direction compared with the ancestral type. The gene clusters between cox2-atp8,
nad3-nad5, nad2-cox1 and control region-nad2 have been identified as the most frequently rearranged regions in mitochondrial genomes of Hymenoptera13,19, which also applied to Trichogramma. A novel tRNA gene cluster trnE-trnF-trnL-trnS1-trnN-trnC formed between cox1 and cox3. The tRNA cluster trnA-trnG formed between two ribosomal RNA genes; this is also novel in the Hymenoptera, in which the trnV gene is typically located between them19; Although the conservation and inversion of large-scale gene blocks in Trichogramma was similar to other sequenced mitochondria genomes of Chalcidoidea, the rearrangement of protein-coding genes nad2 and cox1 as well as most tRNA genes are novel.
Phylogenetic relationships among families of Chalcidoidea. Currently, mitochondrial genomes have been sequenced from three families of Chalcidoidea in seven species. Phylogenetic relationships among the seven species were reconstructed based on protein-coding genes of the mitochondrial genome.

The results showed that the species *Ceratosolen solmsi* from Agaonidae was not clustered with two other species of this family, even when the CAT model was used to avoid among-site rate heterogeneities (Fig. 3A). This species had a long branch compared to other species of ingroup, as shown in the original study of the mitochondrial genome of this species. We predict that the inferred polyphyly of Agaonidae might be caused by long-branch attraction in *C. solmsi*. The Hymenoptera has been shown to be a group with both rapidly and slowly evolving mitochondrial genomes. Long branches have been identified in *Cephalonomia gallicola* (Chrysidoidea: Betylidae), *Wallacidia oculata* (Vespoidea: Mutillidae) and *Primeuchroeus* spp. (Aculeata: Chrysididae).

Identification of other species with long branches may help to improve phylogenetic inference of relationships within Hymenoptera.

By removing the outlier species *C. solmsi* from the analysis, a well-supported phylogenetic relationship among three families of Chalcidoidea was generated (Fig. 3B). The Agaonidae and Pteromalidae formed one lineage, which was then sister to Trichogrammatidae. This is congruent with the currently supported phylogeny of Chalcidoidea.

We also used gene arrangement to reconstruct phylogenetic relationships among the three families. The inferred topology is identical to the one generated from gene sequences (Appendix S1). Our initial work indicates that gene rearrangements of the mitochondrial genome may provide valuable information for recovering phylogenetic relationships within Chalcidoidea. More representative mitochondrial genomes from different groups are needed to validate our assumption.

Ancestral gene order in Chalcidoidea. Large scale gene rearrangements were also found in other sequenced mitochondrial genomes of Chalcidoidea (Fig. 2). Babbucci et al. showed a gene order (GO)
named ant1GO as the plesiomorphic GO for Hymenoptera. Simultaneous rearrangement of large groups of genes has been considered as the initial step of gene rearrangement in the extremely rearranged mitochondrial genomes of *Cotesia vestalis*. Rearrangement of large groups of genes was common in species of Chalcidoidea (Fig. 2). By comparisons, a conserved segment of "trnE-trnF-nad5-trnH-nad4-nad4L-trnT-trnP-nad6-cob" was found among ancestral pattern of insect mitochondrial gene arrangement (PanGO), ant1GO and chalcidoid species of *Megaphragma*, *Philotrypesis* and *Ceratosolen* (Fig. 2), and a segment of "trnS2-nad1-trnL1-rrnL" was shared by ant1GO, PanGO and *Trichogramma* and *Megaphragma* (Fig. 2). Based on the inferred phylogenetic relationships that *Trichogrammatidae* (*Trichogramma* + *Megaphragma*) is sister to (Agaonidae + Pteromalidae) (Fig. 3B), a segment of "trnE-trnF-nad5-trnH-nad4-nad4L-trnT-trnP-nad6-cob-trnS2-nad1-trnL1-rrnL" is more plausible as the ancestral pattern of Chalcidoidea. Within Chalcidoidea, an inverted segment "-cox3-atp6-atp8-trnd-trnk-cox2-trnL2-cox1" compared with PanGO was shared by all analyzed taxa except for *Trichogramma*. A bigger inverted continuous segment with "-nad3-trnG-cox3-atp6-atp8-trnd-trnk-cox2-trnL2-cox1" for Chalcidoidea. For the tRNA clusters, the pattern of "-Dk" was conserved across all analyzed species of Chalcidoidea (Fig. 2). Since there is no conserved pattern in tRNA clusters "trnI-trnQ-trnM", "-trnW-trnC-trnY" and "trnA-trnR-trnN-trnS-trnE-trnF" those in ant1GO were presumed as the ancestral pattern of gene order in Chalcidoidea (ChalcidoidGO in Fig. 4).

Rearrangement pathway of *Trichogramma*. We inferred the evolution of gene arrangement in *Trichogramma* using CREx by comparing the common intervals between ChalcidooidGO and *Trichogramma* gene order (TrichogrammaGO) (Fig. 4). Four operations were considered in CREx, i.e., transpositions, inversions,
inverse transpositions, and TDRL. The CREx identified eight operations in the evolution of gene arrangement in *Trichogramma*, including one transposition (operation 1 in Fig. 4, referring to rrsS), two inverse transpositions (operations 2 and 3 in Fig. 4, referring M and W, respectively), two inversions (operations 4–5 in Fig. 4) and three TDRLs (operations 6–8 in Fig. 4).

There are two sets of alternative scenarios in operations 4–8. The first set of scenarios refers to inversions of *trnI* and tRNA cluster from *trnA* to *trnF*, followed by three TDRLs (operations 4–8 in Fig. 4), while the other set refers to inversion of two large gene segments including both tRNA and protein-coding genes, followed by three TDRLs (operations 4’–8’ in Fig. 4). The presence of intergenic spacers located in the position involved in rearrangements and presence of remnant of the genes that changed positions within intergenic spacers will provide evidence to choose the plausible rearrangement pathways. However, we did not find obvious evidence to support one set of scenarios. Mapping the gene rearrangement patterns on the inferred phylogenetic tree may help to validate the scenarios using MLGO or TreeRex. However, the missing of genes in most species limited the usage of this approach. Rearrangement of tRNA genes are believed to be more frequent than that of large segment. Thus, we presumed that scenarios 4–8 are more plausible than 4’–8’ in rearrangement of *Trichogramma* mitochondrial genomes. However, we could not exclude other pathways due to the computational complexity in gene order reconstruction and the algorithms implemented in CREx.

### Methods

#### DNA extraction.
Specimens of *T. japonicum* and *T. ostriniae* were reared in the Insectary of Nanjing Agricultural University. DNA was extracted from individual wasps using a Wizard® Genomic DNA Purification system (Promega) according to the manufacturer's protocols and stored at −20 °C. The specimens are deposited in the Laboratory of Molecular Ecology and Evolution of Nanjing Agricultural University (*T. ostriniae*: NJAUHymTrich001; *T. japonicum*: NJAUHymTrich003).

#### Mitochondrial genome amplification and annotation.
Two short fragments (518 bp) of the *cox1* gene were amplified using primer set 1718-COI-F/2191-COI-R (Simon et al. 1994) for *T. japonicum and T. ostriniae*. PCR products were purified and sequenced directly using the Sanger method at Shanghai Majorbio Bio-pharm Co., Ltd. (Shanghai, China). According to the obtained sequences, specific PCR primers (Table S4) for each species (*T. japonicum*: Tj-COI-F/Tj-COI-R and *T. ostriniae*: To-COI-F/To-COI-R) were designed to amplify the remaining genome by long PCR as a single fragment, using the manufacturer's rapid PCR protocol. The reaction mixture was composed of 1 μL Tks GfX DNA Polymerase (Takara), 25 μL buffer, 1 μL of each primer (20 μM), 3 μL of DNA with water added to bring a total volume of 50 μL. The cycling conditions were 94 °C for 1 min, 30 cycles of 98 °C for 10 s, 55 °C for 15 s and 68 °C for 10 min. The PCR products were sequenced on an Illumina HiSeq. 2500 platform at Shanghai Majorbio Bio-pharm Co., Ltd. (Shanghai, China). Sequencing libraries for the long PCR fragments were prepared using a TruSeq DNA Sample Prep Kit (Illumina) following the manufacturer's instructions. Libraries were purified with Certified Low Range Ultra Agarose (Bio-Rad), quantified on a TBS380 fluorometer (Invitrogen), pooled and sequenced using a HiSeq SBS Kit V4 with tag sequences generating paired-end reads (read length: 250 bp).

Raw sequencing data were generated by Illumina base calling software CASAVAv1.8.2 (http://support.illumina.com/sequencing/sequencing_software/casava.ilmn), and sequences containing adaptors or primers were identified by SeqPrep (https://github.com/jstjohn/SeqPrep). Sickle (https://github.com/najoshi/sickle) was applied to conduct reads trimming with default parameters to obtain clean data for this study. In addition, SOAPdenovo (http://soap.genomics.org.cn/, v2.05) was used to perform genome assembly with multiple Kmer parameters and assess the assembly results. GapCloser software (downloaded from SOAPdenovo website) was subsequently applied to fill the remaining local inner gaps and correct single-base polymorphisms for the final assembly results.

The tRNA genes were initially identified using tRNA-scan SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/) with the following parameters: source = Mito/Chloromast, and genetic code = Invertebrate Mito. Twenty of the 22 typical animal mitochondrial tRNA genes were identified. The remaining two tRNA and two rRNA genes were confirmed by the MITOS WebServer using invertebrate mitochondrial genetic code (http://mitos.bioinf.uni-leipzig.de/index.py) (Bernt et al., 2013). ORFinder (http://www.ncbi.nlm.nih.gov/GO/orthofinder.cgi) was used to identify protein-coding genes, specifying the invertebrate mitochondrial genetic code.

#### Genome feature analysis.
Base composition, codon usage, Relative Synonymous Codon Usage (RSCU) values and nucleotide substitution were analyzed using MEGA66. GC and AT asymmetries were calculated according to the formulas used in a previous study. AT- and GC-skews were measured for the majority strand using the formulas $\text{AT skew} = (A - T)/(A + T)$ and $\text{GC skew} = (G - C)/(G + C)$. The tandem repeats in the control region were predicted using the Tandem Repeats Finder available online (http://tandem.bu.edu/trf/trf.html) (Bernt et al., 2013).

#### Phylogenetic analysis.
Phylogenetic relationships among three families of Chalcidoidea with sequenced mitochondrial genomes were reconstructed. A phylogenetic tree was reconstructed based on the nucleotide sequences and amino acid sequences of the 13 protein-coding genes. Nucleotide sequences were aligned by codon using MAFFT version 7.205. Phylogenetic relationships were reconstructed with MrBayes version 3.2.5, IQ-TREE web server and PhyloBayes-MPI. In MrBayes analyses, matrices were partitioned by codon position. Then, we used PartitionFinder version 1.1.1 to determine the best partition and substitution models. Four independent Markov chains were run for 10 million metropolis-coupled generations, with tree sampling every 1000 generations and a burn-in of 25%. In IQ-TREE analyses, the number of bootstrap replicates was set to 1000 with CAT model (C20 + 4 G). The CAT-GTR model was applied in PhyloBayes analyses with independent Markov chain runs of 8000 and a burn-in of 1000 and subsample of 10 trees. *Pelecinus polyturator* (Proctotrupoida:...
Pelecinidae) and Vanhornia eucnemidarum (Proctotrupoidea: Vanhorniidae) were chosen as outgroups according to previously inferred phylogenetic relationships among major lineages of Apocrita.

We also inferred phylogenetic relationships among the three families of Chalcidoidea based on gene arrangement patterns. Phylogenetic relationships were inferred using a Maximum Likelihood (ML) method based on gene-order data implemented in the MLGO web server. We excluded C. solmsi from the analysis due to missing genes and the inclusion of other representatives from the family Agaonidae.

**Gene organization analysis.** The evolutionary pathways of gene arrangement in Trichogramma were estimated by CREx (Common Interval Rearrangement Explorer). The heuristic method based on common interval was used to determine genome rearrangement scenarios between presumed ancestral gene order of Chalcidoidea and that of Trichogramma. Gene rearrangement pattern were mapped to the phylogenetic tree using MLGO web server. The ChalcidoidGO was used as outgroup.

**Data availability statement.** The data were deposited into GenBank under accession numbers: KU577436 and KU577437.

**Conclusion**

The two mitochondrial genomes sequenced in our study from Trichogramma add to our knowledge of the mitochondrial genomes of Hymenoptera. Compared with those of other related insects, the mitochondrial genomes of Trichogramma were significantly rearranged, not only in tRNA genes but also in many protein-coding genes. Congruent tree topologies were recovered using gene sequences, including nucleotides and amino acids. The specific gene order in mitochondrial genomes of these species indicated that gene rearrangement information may represent potentially valuable data for phylogenetic analyses of Chalcidoidea. The availability of the complete mitochondrial genomes of Trichogramma provides information for development of genetic markers to study community ecology, population biology and evolution in this species complex.

**References**

1. Wolstenholme, D. R. Animal mitochondrial DNA: Structure and evolution. *Int Rev Cytol* **141**, 173–216 (1992).
2. Dowton, M., Belshaw, R., Austin, A. D. & Quicke, D. L. J. Simultaneous molecular and morphological analysis of Braconid relationships (Insecta: Hymenoptera: Braconidae) indicates independent mt-tRNA gene inversions within a single wasp family. *J Mol Evol* **54**, 210–226 (2002).
3. Cameron, S. L. Insect mitochondrial genomics: Implications for evolution and phylogeny. *Annu Rev Entomol* **59**, 95–117 (2014).
4. Booze, J. L. & Brown, W. M. Big trees from little genomes: mitochondrial gene order as a phylogenetic tool. *Curr Opin Genet Dev* **8**, 668–674 (1998).
5. Li, Q. et al. Multiple lines of evidence from mitochondrial genomes resolve phylogenetic relationships of parasitic wasps in Braconidae. *Genome Biol. Evol* **8**, 2561-2562 (2016).
6. Mao, M., Austin, A. D., Johnson, N. F. & Dowton, M. Coexistence of minicircular and a highly rearranged mtDNA molecule suggests that recombination shapes mitochondrial genome organization. *Mol Biol Evol* **31**, 636–644 (2014).
7. Shao, R., Campbell, N. J. H., Schmidt, E. R. & Barker, S. C. Increased rate of gene rearrangement in the mitochondrial genomes of three orders of hemipteroid insects. *Mol Biol Evol* **18**, 1828–1832 (2001).
8. Booze, J. L. Animal mitochondrial genomes. *Nucleic Acids Research* **27**(1714), 1767–1780 (1999).
9. Covacin, C., Shao, R., Cameron, S. & Barker, S. C. Extraordinary number of gene rearrangements in the mitochondrial genomes of lice (Phthiraptera: Insecta). *Insect Mol Biol* **15**, 63–68 (2006).
10. Cameron, S. L., Yoshizawa, K., Mizukoshi, A., Whiting, M. F. & Johnson, K. P. Mitochondrial genome deletions and minicircles are common in lice (Insecta: Phthiraptera). *BMCGenomics* **12**, 394 (2011).
11. Cameron, S. L., Johnson, K. P. & Whiting, M. F. The mitochondrial genome of the louse *Botheriotomus solmsi* (Phthiraptera: Ischnocera): effects of extensive gene rearrangements on the evolution of the genome. *J Mol Evol* **65**, 589–604 (2007).
12. Chen, W. J. et al. The mitochondrial genome of Sinentomon erythraeum (Arthropoda: Hexapoda: Protura): An example of highly divergent evolution. *BMCGenomics* **11**, 246 (2011).
13. Wei, S. J., Li, Q., van Achterberg, K. & Chen, X. X. Two mitochondrial genomes from the families Bethylidae and Mutillidae: independent rearrangement of protein-coding genes and higher-level phylogeny of the Hymenoptera. *Molecular Phylogenetics and Evolution* **77**, 1–10 (2014).
14. Xiao, J. H., Ji, J. G., Murphy, R. W. & Huang, D. W. Rapid evolution of the mitochondrial genome in chalcidoid wasps (Hymenoptera: Chalcidoidea) driven by parasitic lifestyles. *Plos One* **6**, e26645 (2011).
15. Wei, S. J., Shi, M., Sharkey, M. J., van Achterberg, C. & Chen, X. X. Comparative mitogenomics of Braconidae (Insecta: Hymenoptera) and the phylogenetic utility of mitochondrial genomes with special reference to holometabolous insects. *BMCGenomics* **11**, 371 (2010).
16. Dowton, M., Cameron, S. L., Dowavic, J. I., Austin, A. D. & Whiting, M. F. Characterization of 67 mitochondrial tRNA gene rearrangements in the Hymenoptera suggests that mitochondrial tRNA gene position is selectively neutral. *Mol Biol Evol* **26**, 1607–1617 (2009).
17. Sankoff, D. et al. Gene order comparisons for phylogenetic inference: evolution of the mitochondrial genome. *Proceedings of the National Academy of Sciences, USA* **89**, 6575–6579 (1992).
18. Booze, J. L., Lavrov, D. V. & Brown, W. M. Gene translocation links insects and crustaceans. *Nature* **392**, 667–668 (1998).
19. Mao, M., Gibson, T. & Dowton, M. Evolutionary dynamics of the mitochondrial genome in the evaniomorpha (hymenoptera)-a group with an intermediate rate of gene rearrangement. *Genome Biol. Evol* **6**, 1862–1874 (2014).
20. Dowton, M. & Austin, A. D. Increased genetic diversity in mitochondrial genes is correlated with the evolution of parasitism in the Hymenoptera. *J Mol Evol* **41**, 958–965 (1995).
21. Shao, R., Campbell, N. J. H. & Barker, S. C. Numerous gene rearrangements in the mitochondrial genome of the wallaby louse, *Heterodoxus macropus* (Phthiraptera). *Mol Biol Evol* **18**, 858–865 (2001).
22. Shao, R. & Barker, S. C. Rates of gene rearrangement and nucleotide substitution are correlated in the mitochondrial genomes of insects. *Mol Biol Evol* **20**, 1612–1619 (2003).
23. Baldacci, M., Basso, A., Scupola, A., Patarnello, T. & Negrissolo, E. Is it an ant or a butterfly? Convergent evolution in the mitochondrial gene order of Hymenoptera and Lepidoptera. *Genome Biol. Evol* **6**, 3326–3343 (2014).
24. Dowton, M., Castro, L. R., Campbell, S. L., Bargon, S. D. & Austin, A. D. Frequent mitochondrial gene rearrangements at the Hymenopteran nad3-nad5 junction. *J Mol Evol* **56**, 517–526 (2003).
25. Dowton, M. & Austin, A. D. Evolutionary dynamics of a mitochondrial rearrangement "hot spot" in the Hymenoptera. Mol Biol Evol 16, 298–309 (1999).

26. Dowton, M., Castro, L. R. & Austin, A. D. Mitochondrial gene rearrangements as phylogenetic characters in the invertebrates: the examination of genome 'morphology'. Invertbr Syst 16, 345–356 (2002).

27. Bernt, M. et al. CREx: inferring genomic rearrangements based on common intervals. Bioinformatics 23, 2957–2958 (2007).

28. Wei, S. J., Wu, Q. L., van Achterberg, K. & Chen, X. X. Rearrangement of the nad1 gene in Pristaulacus compressus (Spinola) (Hymenoptera: Eupelmidae) mitochondrial genome. Mitochondrial DNA 25, 629–630 (2015).

29. Hu, Q. L. et al. The complete mitochondrial genome of Taeniogonolas taitohirana (Bischoff) (Hymenoptera: Trigonalyidae) reveals a novel gene rearrangement pattern in the Hymenoptera. Gene 543, 76–84 (2014).

30. Oliveira, D. C. S. G., Raychoudhury, R., Lavrov, D. V. & Werren, J. H. Rapidly evolving mitochondrial genome and directional selection in mitochondrial genes in the parasitic wasp Nasonia (Hymenoptera: Pteromalidae). Mol Biol Evol 25, 2167–2180 (2008).

31. Nelduzhojko, A. V. et al. Mitochondrial genome of Megaphragma amblyanthum (Hymenoptera: Trichogrammatidae). Mitochondrial DNA, https://doi.org/10.3109/19401736.19402015.1101546 (2015).

32. Dowton, M., Cameron, S. L., Austin, A. D. & Whiting, M. F. Phylogenetic approaches for the analysis of mitochondrial genome sequence data in the Hymenoptera – A lineage with both rapidly and slowly evolving mitochondrial genomes. Mol Phylogenet Evol 52, 512–519 (2009).

33. Mao, M. & Dowton, M. Complete mitochondrial genomes of Ceratobaeus sp. and Idris sp. (Hymenoptera: Scelionidae): shared gene rearrangements as potential phylogenetic markers at the tribal level. Mol Biol Rep 41, 6419–6424 (2014).

34. Wei, S. J., Shi, M., He, J. H., Sharkey, M. J. & Chen, X. X. The complete mitochondrial genome of Diaegema semicalausum (Hymenoptera: Ichneumonidae) indicates extensive independent evolutionary events. Genome 52, 308–319 (2009).

35. Machida, R. J., Miya, M. U., Nishida, M. & Nishida, S. Complete mitochondrial DNA sequence of Tigrionus japonicus (Crustacea: Copepoda). Mar Biotechnol 4, 406–417 (2002).

36. Ito, A., Aoki, M. N., Yokobori, S. & Wada, H. The complete mitochondrial genome of Caprella scabra (Crustacea, Amphipoda, Caprellidae), with emphasis on the unique gene order pattern and duplicated control region. Mitochondrial DNA 21, 183–190 (2010).

37. Crozier, R. H. & Crozier, Y. C. The mitochondrial genome of the honeybee Apis mellifera: complete sequence and genome organization. Genetics 133, 97–117 (1993).

38. Cameron, S. L. et al. Mitochondrial genome organization and phylogeny of two vespid wasps. Genome 51, 800–808 (2008).

39. Hassani, A., Léger, N. & Deutsch, J. Evidence for multiple reversals of asymmetric mutational constraints during the evolution of the mitochondrial genome of metazoan, and consequences for phylogenetic inferences. Systematic Biology 54, 277–298 (2015).

40. Wei, S. J. et al. New views on strand asymmetry in insect mitochondrial genomes. PloS One 5, e12708 (2010).

41. Perne, N. T. & Kocher, T. D. Patterns of nucleotide composition at fourfold degenerate sites of animal mitochondrial genomes. J Mol Biol 41, 353–358 (1995).

42. Hasegawa, E., Kobayashi, K., Yagi, N. & Tsuji, K. Complete mitochondrial genomes of normal and cheater morphs in the parasitogenic wasp Pristaulacus compressus (Spinola) (Hymenoptera: Eupelmidae). Mitochondrial DNA 27, 2653–2655 (2016).

43. Lavrov, D. V., Brown, W. M. & Boore, J. L. A novel type of RNA editing occurs in the mitochondrial tRNAs of the centipede Lithobius forficatus. Proc Natl Acad Sci USA 97, 13738–13742 (2000).

44. Korkmaz, E. M., Dogan, O., Budak, M. & Basıbüyük, H. H. Two nearly complete mitogenomes of wheat stem borers, Cephus pygmeus (L.) and Cephus sareptanus Dovnar-Zapolskij (Hymenoptera: Cephidae): An unusual elongation of rnrS gene. Gene 558, 254–264 (2015).

45. Foster, P. G., Jermiin, L. S. & Hickey, D. A. Nucleotide composition bias affects amino acid content in proteins coded by animal mitochondria. J Mol Evol 44, 282–288 (1997).

46. Zhou, Z. J., Huang, Y. & Shi, F. M. The mitochondrial genome of Ruspalia dubia (Orthoptera: Conocephalidae) contains a short A + T-rich region of 70 bp in length. Genome 50, 855–866 (2007).

47. Wei, S. J., Pang, P., Zheng, L. H., Shi, M. & Chen, X. X. The complete mitochondrial genome of Evania appendigaster (Hymenoptera: Eupelmidae) has low A + T content and a long intergenic spacer between atp8 and atp6. Molecular Biology Reports 37, 1931–1942 (2010).

48. Zhang, D. X. & Hewitt, G. M. Insect mitochondrial control region: A review of its structure, evolution and usefulness in evolutionary studies. Biochem Syst Ecol 25, 99–120 (1997).

49. Cameron, S. L. & Whiting, M. F. Mitochondrial genomic comparisons of the subterranean termites from the Genus Zootermopsis. Zool Scr, 179–190 (2015).

50. Mou, M., Tan, X., Xie, Y. & F. M. The complete mitochondrial genome of the Plague Thrips, Thrips imaginis (Thysanoptera: Thripidae) reveals a novel gene rearrangement pattern in the Hymenoptera. Proc Natl Acad Sci USA 97, 13738–13742 (2000).

51. Clary, D. O. & Wolstenholme, D. R. The mitochondrial DNA molecule of Drosophila yakuba: Nucleotide sequence, gene organization, and genetic code. J Mol Biol 222, 252–271 (1985).

52. Korkmaz, E. M., Dogan, O., Budak, M. & Basıbüyük, H. H. Two nearly complete mitogenomes of wheat stem borers, Cephus pygmeus (L.) and Cephus sareptanus Dovnar-Zapolskij (Hymenoptera: Cephidae): An unusual elongation of rnrS gene. Gene 558, 254–264 (2015).

53. Foster, P. G., Jermiin, L. S. & Hickey, D. A. Nucleotide composition bias affects amino acid content in proteins coded by animal mitochondria. J Mol Evol 44, 282–288 (1997).

54. Zhou, Z. J., Huang, Y. & Shi, F. M. The mitochondrial genome of Ruspalia dubia (Orthoptera: Conocephalidae) contains a short A + T-rich region of 70 bp in length. Genome 50, 855–866 (2007).

55. Wei, S. J., Pang, P., Zheng, L. H., Shi, M. & Chen, X. X. The complete mitochondrial genome of Evania appendigaster (Hymenoptera: Eupelmidae) has low A + T content and a long intergenic spacer between atp8 and atp6. Molecular Biology Reports 37, 1931–1942 (2010).

56. Zhang, D. X. & Hewitt, G. M. Insect mitochondrial control region: A review of its structure, evolution and usefulness in evolutionary studies. Biochem Syst Ecol 25, 99–120 (1997).

57. Cameron, S. L. & Whiting, M. F. Mitochondrial genomic comparisons of the subterranean termites from the Genus Zootermopsis. Zool Scr, 179–190 (2015).

58. Mou, M., Tan, X., Xie, Y. & F. M. The complete mitochondrial genome of the Plague Thrips, Thrips imaginis (Thysanoptera: Thripidae) reveals a novel gene rearrangement pattern in the Hymenoptera. Proc Natl Acad Sci USA 97, 13738–13742 (2000).

59. Clary, D. O. & Wolstenholme, D. R. The mitochondrial DNA molecule of Drosophila yakuba: Nucleotide sequence, gene organization, and genetic code. J Mol Biol 222, 252–271 (1985).

60. Korkmaz, E. M., Dogan, O., Budak, M. & Basıbüyük, H. H. Two nearly complete mitogenomes of wheat stem borers, Cephus pygmeus (L.) and Cephus sareptanus Dovnar-Zapolskij (Hymenoptera: Cephidae): An unusual elongation of rnrS gene. Gene 558, 254–264 (2015).

61. Korkmaz, E. M., Dogan, O., Budak, M. & Basıbüyük, H. H. Two nearly complete mitogenomes of wheat stem borers, Cephus pygmeus (L.) and Cephus sareptanus Dovnar-Zapolskij (Hymenoptera: Cephidae): An unusual elongation of rnrS gene. Gene 558, 254–264 (2015).
66. Ronquist, F. et al. MrBayes 3.2: efficient bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**, 539–542 (2012).
67. Nguyen, L. T., Schmidt, H. A., von Haeseler, A. & Minh, B. Q. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular biology and evolution* **32**, 268–274 (2015).
68. Lartillot, N., Lepage, T. & Blanquart, S. PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating. *Bioinformatics* **25**, 2286–2288 (2009).
69. Lanfear, R., Calcott, B., Ho, S. Y. & Guindon, S. Partitionfinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* **29**, 1695–1701 (2012).
70. Lartillot, N. & Philippe, H. A Bayesian mixture model for across-site heterogeneities in the amino-acid replacement process. *Mol Biol Evol* **21**, 1095–1109 (2004).
71. Castro, L. R., Ruberu, K. & Dowton, M. Mitochondrial genomes of *Vanhornia eucnemidarum* (Apocrita: Vanhorniidae) and *Primeuchroeus* spp. (Aculeata: Chrysididae): Evidence of rearranged mitochondrial genomes within the Apocrita (Insecta: Hymenoptera). *Genome* **49**, 752–766 (2006).

**Acknowledgements**

This research was funded by the National Natural Science Foundation of China (31472025, 31101661), National Basic Research Program of China (Grant No. 2013CB127600) and Special Fund for Agro-scientific Research of China (201203036).

**Author Contributions**

Yuan-Xi Li and Shu-Jun Wei conceived and designed the experiments; Long Chen, Xiao-Feng Xue and Hai-Qing Hua performed the experiments; Shu-Jun Wei, Long Cheng and Peng-Yan Chen analyzed the data; Long Chen, Yuan-Xi Li, Shu-Jun Wei wrote the paper. Yuan-Xi Li, Shu-Jun Wei and Fan Zhang discussed the results. All authors reviewed the manuscript.

**Additional Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-25338-3.

**Competing Interests:** The authors declare no competing interests.

**Publisher's note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018