The Complete Mitochondrial Genomes of Two Octopods
*Cistopus chinensis* and *Cistopus taiwanicus*: Revealing the Phylogenetic Position of the Genus *Cistopus* within the Order Octopoda

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**Abstract**

In the present study, we determined the complete mitochondrial DNA (mtDNA) sequences of two species of *Cistopus*, namely *C. chinensis* and *C. taiwanicus*, and conducted a comparative mt genome analysis across the class Cephalopoda. The mtDNA length of *C. chinensis* and *C. taiwanicus* are 15706 and 15793 nucleotides with an AT content of 76.21% and 76.5%, respectively. The sequence identity of mtDNA between *C. chinensis* and *C. taiwanicus* was 88%, suggesting a close relationship. Compared with *C. taiwanicus* and other octopods, *C. chinensis* encoded two additional tRNA genes, showing a novel gene arrangement. In addition, an unusual 23 poly (A) signal structure is found in the ATP8 coding region of *C. chinensis*. The entire genome and each protein coding gene of the two *Cistopus* species displayed notable levels of AT and GC skews. Based on sliding window analysis among Octopodiformes, ND1 and DN5 were considered to be more reliable molecular beacons. Phylogenetic analyses based on the 13 protein-coding genes revealed that *C. chinensis* and *C. taiwanicus* form a monophyletic group with high statistical support, consistent with previous studies based on morphological characteristics. Our results also indicated that the phylogenetic position of the genus *Cistopus* is closer to *Octopus* than to *Amphioctopus* and *Callistoctopus*. The complete mtDNA sequence of *C. chinensis* and *C. taiwanicus* represent the first whole mt genomes in the genus *Cistopus*. These novel mtDNA data will be important in refining the phylogenetic relationships within Octopodiformes and enriching the resource of markers for systematic, population genetic and evolutionary biological studies of Cephalopoda.

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**Introduction**

Cephalopods are the most intelligent, mobile and the largest of all mollusks, with all members marine. Two groups of cephalopods exist today: *Nautiloidea* with a few species of the pearly nautilus, and *Coleoidea*, containing the squids, cuttlefishes, octopods and vampire squids [1]. Octopods are arguably one of the most charismatic cephalopods, because of their importance as fisheries resources, reported intelligence and behavioural complexity, vertebrate-like eyes and well-developed capabilities for rapid change in appearance. Although octopods display a wide diversity in skin coloration, behaviour and life strategies, they bear a strong similarity in structural morphology. Furthermore, in stark contrast to most other mollusks, octopods lack substantial hard parts with sufficient morphological characters that can be used in determining phylogenetic relationships [2]. Even in comparison with the squids and cuttlefishes, soft bodies of octopods often preserve poorly and frequently distort after death and preservation. As a consequence, higher-level systematic relationships within the octopod group, species limits and identification are difficult to establish. Therefore, besides morphological characters, molecular techniques should be applied to increase the accuracy of phylogenetic relationship assessments between octopods.

Most metazoan species possess a compact, circular mitochondrial (mt) genome, which varies in size from 14 to 19 kb that typically contains 37 genes, including 13 protein coding
genes, two ribosomal RNAs (rRNA) genes and 22 transfer RNAs (tRNA) genes necessary for translation of the proteins encoded by the mtDNA [3,4]. mtDNA has been extensively used for studying phylogenetic and evolutionary relationships among animal species, due to its maternal inheritance, rapid evolutionary rate, and lack of genetic recombination [5-7]. Partial sequences of mtDNA genes, such as cytochrome oxidase I (CO1), cytochrome oxidase III (CO3) and 16S rRNA, have proved to be an important tool in intra-specific and inter-specific phylogenetic studies of Cephalopoda and other mollusks. Compared to partial mt genes, complete mtDNA sequence can uncover more information about gene rearrangement and other variation at the genome level for all phyla, and are especially powerful for displaying sufficient interspecies sequence variability and describing species specificity [3]. However, up to now, only three complete mt genomes of octopods have been determined: Octopus vulgaris, Octopus minor and Amphioctopus fangsiao [3,8,9]. Thus, additional complete mtDNA sequences of are urgently needed to resolve the taxonomic problems in octopods. The genus Cistopus (family Octopodidae) was erected by Gray (1849) based on the presence of eight small pouches in the web between the bases of each arm. This genus is common in the Indo-Malayan area [10]. The eight pouches contain mucus, which can be released through a small muscular pore opening to the exterior between the proximal suckers [11]. Cistopus indicus was mistakenly recognized as the sole species in this genus for a long time and the name C. indicus has been applied to all specimens found in the area of southern China, Taiwan, the Philippines, northern Indonesia and west to India. In 1997, Norman and Nateewathana first mentioned the existence of additional taxa of the genus Cistopus [10,12]. Recently, two new species in the genus, C. chinensis and C. taiwanicus, were identified [13,14]. The newly identified Cistopus species enriched our knowledge of Cistopus and contributed to our understanding of evolution in the family Octopodidae. However, there has been almost no molecular information about the two newly identified Cistopus species, failing to determine the phylogenetic position of the genus Cistopus. In addition, the mucous pouches are often difficult or even impossible to see in preserved specimens, resulting in regular misidentifications. Therefore, specific PCR primers for Cistopus would be useful in order to provide tools that could differentiate Cistopus from other morphologically similar octopods. In this study, we determined the complete nucleotide sequences of C. chinensis and C. taiwanicus and compared the sequences with other cephalopod mt genomes. The new mtDNA sequences may provide useful information on both genomics and the evolution of octopods, because there are only three complete mtDNA sequences available from Octopoda. Furthermore, the new mtDNA information can help determine the position of Cistopus in the family Octopodidae.

Results and Discussion

General features of the mt genomes

The complete mt genomes of C. chinensis and C. taiwanicus are circular molecules with 15706 bp and 15793 bp, respectively. The two Cistopus species showed 88% sequence identities in mtDNA sequences, indicating a close relationship with each other. The C. taiwanicus mitogenome contained the typical 37 genes (13 protein-coding genes, 22 tRNA genes, and 2 rRNA genes), while the C. chinensis mitogenome encoded 39 genes with 2 rRNA genes (tRNA-Phe1 and tRNA-Leu3) additional to the normal complement (Figure 1). The mitochondrial genome sizes of the two Cistopus are similar to the published mitochondrial genomes of other Octopodiformes species. These genomes range from 15617 bp (Vampyroteuthis infernalis) to 15979 bp (Amphioctopus fangsiao) [8,15]. Difference in genome size is usually due to the variation of intergenetic regions and the presence of hypothetical proteins. Overall, the mitochondrial genomes of Octopodiformes are highly compact, with over 90% of the genome encoding for structural genes. Overlapping of adjacent genes is also common in many animals’ mt genomes, although the extent of overlaps varies [16,17]. In C. taiwanicus, gene overlaps were observed at 19 gene junctions and involved a total of 329 bp, whereas in C. chinensis, gene overlaps were observed at 22 gene junctions and involved a total of 424 bp. As shown in Table 1, the longest overlaps in C. chinensis and C. taiwanicus were 155 bp (between ND2 and CO1) and 72 bp (between ND1 and tRNA-Leu1), respectively. Interestingly, the tRNA-Leu1 gene is found to be completely located within ND1 in C. taiwanicus. In addition to the long noncoding region, the mitochondrial genomes of C. chinensis and C. taiwanicus contained 7 and 12 intergenetic spacers, respectively, ranging from 1 to 65 bp in length (Table 1). The longest spacer sequences in C. chinensis and C. taiwanicus were located between CO3 and tRNA-Lys, which were 65 and 30 bp, respectively.

Genome Composition and Gene Order Analysis of mt Genomes

The nucleotide compositions of the complete mtDNA sequences of C. chinensis and C. taiwanicus are biased toward A and T, with A being the most favored nucleotide and G the least favored, in accordance with the mt genomes of other reported cephalopods (Table 2). The content of A+T is 76.21% for C. chinensis (41.14% A, 35.07% T, 7.34% G and 16.45% C) and 76.50% for C. taiwanicus (41.35% A, 35.15% T, 7.48% G and 16.01% C) respectively. Strand asymmetry is usually reflected by skewness, which is calculated as (A-T)/(A+T) and (G-C)/(G+C), respectively. AT-skews and GC-skews of the whole mt genome were calculated for 24 cephalopods species to date (Table 2). This composition of full mtDNA sequence of C. chinensis and C. taiwanicus is strongly skewed away from T in favor of A. The pattern of skew of the two Cistopus species is highly congruent with that observed in the mtDNA sequences of other cephalopods (Table 2). GC skew is suggested as the best indicator of strand asymmetry according to previous studies [18]. As shown in Table 2, the cephalopod species analyzed in the present study showed obvious strand asymmetry (GC skew between -0.266 and -0.412). The GC skew of C. chinensis and C. taiwanicus is -0.382 and -0.363, respectively. Interestingly, in all mt genome sequences of cephalopods reported to date, the GC skew is negative due to
the significantly low G content in mt genomes. In mammals, these asymmetric and biased base composition of mt genomes may be due to the spontaneous domination process of C and A in the H-strand during replication [19].

Gene order is generally conserved in most taxa, although some groups show considerable variation. This is particularly so in the Phylum Mollusca, especially for the cephalopods [9,20]. In Cephalopoda, the gene arrangements of protein-coding and tRNA genes are highly diversified. The gene arrangement of the two Cistopus species clearly differs. C. taiwanicus has a typical gene arrangement, identical to that of the three previously sequenced octopus species and V. infernalis (Figure 1). However, C. chinensis exhibits a novel gene arrangement, including 13 protein coding genes, 2 rRNAs and 24 tRNAs. The two additional tRNAs appear to specify phenylalanine and leucine, making the gene order of C. chinensis mt genome significantly different from that of any octopod or cephalopod reported to date. Although additional tRNA genes have been reported in several mtDNA genomes, its function remains unclear [21,22].

**Protein-coding genes**

Both C. chinensis and C. taiwanicus mtDNAs contained 13 typical protein-coding genes. The H-strand and L-strand of two mitochondrial genomes of C. taiwanicus and C. chinensis have been designated according to the molecular weight of the
Table 1. Positions and nucleotide lengths of the mitochondrial genomes of *Cistopus chinensis* (CH) and *Cistopus taiwanicus* (CW).

| Gene | Strand | Gene position       | Initiation/Stop condon | anticodon | Intergenic nucleotides |
|------|--------|---------------------|------------------------|-----------|------------------------|
|      |        | CH      | TW      | CH  | TW | CH | TW |
| CO3  | H      | 1-780   | 1-780   | ATG | TAA | ATG | TAA | 65 | 30 |
| tRNA-Lys | H   | 846-916 | 810-877 | TTT | -3 | -3 |
| tRNA-Ala   | H    | 914-983 | 875-944 | TGC | -2 | -2 |
| tRNA-Arg   | H    | 882-1047| 943-1007| TCG | -3 | -1 |
| tRNA-Asn   | H    | 1045-1118| 1007-1077| GTT | -3 | 1  |
| tRNA-Ile   | H    | 1116-1184| 1078-1146| GAT | -25 | -66 |
| ND3      | H    | 1160-1534| 1081-1497| ATA | TAA | ATA | TAA | -1 | -2 |
| tRNA-Ser2 | H    | 1534-1600| 1496-1564| GCT | -17 | -18 |
| ND2      | H    | 1584-2768| 1547-2731| ATA | TAA | ATA | TAA | -155 | -59 |
| CO1      | H    | 2614-4146| 2673-4109| ATG | TAA | ATA | TAA | 4  | 2  |
| CO2      | H    | 4151-4837| 4111-4797| ATG | TAA | ATG | TAA | -68 | -3 |
| tRNA-Phe1 | L    | 4770-4842| —       | AAA | -7 | - |
| tRNA-Asp | H    | 4836-4902| 4795-4864| GTC | 1  | 1  |
| ATP8     | H    | 4904-5053| 4685-5020| ATG | TAA | ATG | TAA | -10 | -11 |
| ATP6     | H    | 5044-5739| 5010-5714| ATA | TAG | ATA | TAG | 15  | 26  |
| tRNA-Phe2 | L   | 5755-5814| 5740-5806| GAA | -12 | -53 |
| ND5      | L    | 5803-7530| 5754-7499| ATA | TAA | ATG | TAA | -16 | -1 |
| tRNA-His | L    | 7515-7581| 7499-7565| GTG | 4  | 4  |
| ND4      | L    | 7586-8929| 7569-8912| ATA | TAA | ATA | TAA | -4  | -4  |
| ND4L     | L    | 8926-9231| 8909-9214| ATA | TAG | ATA | TAG | -5  | -5  |
| tRNA-Thr | H    | 9227-9291| 9210-9275| TGT | 2  | 3  |
| tRNA-Ser1 | L   | 9294-9370| 9278-9350| TGA | -13 | -10 |
| Cytb     | L    | 9358-10482| 9341-10486| ATA | TAG | ATA | TAG | -14 | -14 |
| ND6      | L    | 10469-11002| 10473-10985| ATA | TAG | ATG | -20 | 1  |
| tRNA-Pro  | L   | 10983-11049| 10986-11054| TGG | 0  | 7  |
| ND1      | L    | 11050-12024| 11061-12074| ATA | TAG | ATA | TAA | -31 | -72 |
| tRNA-Leu1 | L   | 11994-12066| 12002-12073| TAA | 2  | 2  |
| tRNA-Leu2 | L   | 12065-12131| 12072-12138| TAG | 0  | 0  |
| 16S      | L    | 12132-13463| 12139-13549| TAC | 0  | 0  |
| tRNA-Val | L    | 13464-13531| 13550-13620| TAC | 0  | 0  |
| 12S      | L    | 13532-14526| 13621-14610| TAC | 0  | 0  |
| tRNA-Met  | L   | 14527-14598| 14611-14680| CAT | 4  | 4  |
| tRNA-Cys  | L   | 14603-14665| 14685-14746| GCA | -10 | -2 |
| tRNA-Tyr  | L   | 14656-14729| 14745-14811| GTA | 0  | 0  |
| tRNA-Trp  | L   | 14730-14795| 14812-14877| TCA | 0  | -1 |
| tRNA-Gln  | L   | 14796-14863| 14877-14946| TTG | 0  | 1  |
| tRNA-Gly  | L   | 14864-14930| 14947-15011| TCC | -3 | 1  |
| tRNA-Glu  | L   | 14928-14999| 15012-15081| TTC | 513 | 712 |
| tRNA-Leu3 | L   | 15513-15582—| TAA | 124 | - |

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bases (Figure 1). In the two *Cistopus* species, seven of the thirteen proteins are encoded by the H-strand, while the other six proteins are encoded by the L-strand (Table 1). The amino acid numbers of predicted *mt* proteins in *C. chinensis* and *C. taiwanicus* were 3793 and 3799, respectively, which were slightly higher than that of other Octopodiformes species [3]. Mitochondrial genomes often use a variety of nonstandard initiation codons. In *C. chinensis*, ND3, ND2, APT6, ND5, ND4, ND4L, Cybt and ND6 initiate with ATG as start codon, while the other five proteins start with the standard ATG. Ten of the thirteen proteins use TAA as stop codon, and the remaining four genes terminate with TAG in *C. chinensis* (Table 1). While in *C. taiwanicus*, five of thirteen protein-coding genes initiate with ATG as start codon, ND3, ND2, CO1 APT6, ND4, ND4L, Cybt and ND1 start with ATA. Nine protein-coding genes use TAA as stop codon, and the remaining four genes terminate with TAG (Table 1). It is worth noting that there is an unusual 23 poly (A) signal structure in the ATP8 coding region of *C. chinensis*, whereas the length of poly (A) signal structure in *C. taiwanicus* and other Octopodiformes species is less than 9.

CO2 and APT8 exhibit positive AT skew in the two *Cistopus* species, while the other eleven protein-coding genes show a
Table 2. Nucleotide composition of the mitochondrial genomes of Cephalopods species.

| Species              | Composition | AT content | Length | AT skew | GC skew |
|----------------------|-------------|------------|--------|---------|---------|
|                      | A  | C  | G  | T  |        |          |        |
| Cistopus chinensis   | 6461 | 2584 | 1153 | 5508 | 76.21% | 15706   | 0.07962 | -0.38293 |
| Cistopus t aiwanicus | 6531 | 2529 | 1182 | 5551 | 76.50% | 15753   | 0.08111 | -0.36297 |
| Octopus vulgaris     | 6478 | 2764 | 1193 | 5309 | 74.87% | 15744   | 0.09918 | -0.39702 |
| Amphiocopus fangsiou | 6758 | 2473 | 1175 | 5573 | 77.17% | 15979   | 0.0961  | -0.35581 |
| Octopus minor        | 6492 | 2683 | 1227 | 5572 | 75.52% | 15974   | 0.07626 | -0.37238 |
| Vampyroteuthis infernalis | 6331 | 2254 | 1183 | 5869 | 78.12% | 15617   | 0.03787 | -0.31929 |
| Sepia officinalis    | 6188 | 2880 | 1534 | 5561 | 72.69% | 16163   | 0.05337 | -0.30494 |
| Sepia esculenta      | 6367 | 2765 | 1530 | 5537 | 73.49% | 16199   | 0.06972 | -0.28754 |
| Sepioteuthis lessoniana | 6184 | 3216 | 1577 | 5654 | 71.18% | 16631   | 0.04477 | -0.34196 |
| Sepiella japonica    | 6457 | 2677 | 1301 | 5737 | 75.40% | 16172   | 0.05905 | -0.3459  |
| Sepia pharaonis      | 6642 | 2380 | 1298 | 5886 | 77.32% | 16208   | 0.05905 | -0.3459  |
| Sepiella inermis      | 6476 | 2653 | 1295 | 5767 | 75.62% | 16191   | 0.06018 | -0.29418 |
| Seminorrosia patagonica | 6974 | 2760 | 1318 | 6034 | 76.13% | 17066   | 0.07226 | -0.3536  |
| Loligo bleekeri      | 6679 | 3356 | 1588 | 5588 | 71.27% | 17211   | 0.08894 | -0.35761 |
| Doryteuthis opalescens | 6730 | 3386 | 1659 | 5612 | 70.98% | 17387   | 0.09059 | -0.34232 |
| Watasenia scintillans | 7083 | 3843 | 2336 | 6831 | 69.25% | 20093   | 0.01811 | -0.24389 |
| Todarodes pacificus   | 7783 | 3547 | 1998 | 6926 | 72.62% | 20254   | 0.05826 | -0.27935 |
| Architecthia dux      | 8010 | 4242 | 1914 | 6165 | 69.72% | 20331   | 0.13016 | -0.37817 |
| Dosidicus gigas       | 7579 | 4117 | 2118 | 6510 | 69.32% | 20324   | 0.07588 | -0.30261 |
| Sthenoteuthis oualaniensis | 7246 | 3889 | 2384 | 6787 | 69.11% | 20306   | 0.03271 | -0.23992 |
| Ommastrephes bartramii | 6803 | 4576 | 2653 | 6274 | 64.39% | 20308   | 0.04045 | -0.26601 |
| Bathyeuthis abyssicola | 7982 | 3539 | 1865 | 6688 | 73.08% | 20075   | 0.08821 | -0.30977 |
| Nautilus macromphalus | 5486 | 4639 | 1932 | 4201 | 59.58% | 16258   | 0.13265 | -0.41196 |

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Typical negative AT skew (Table S1). In C. t aiwanicus, the AT skew value for CO2 and APT8 is 0.01 and 0.06, respectively, while in C. ch inensis, it is 0.02 and 0.11, respectively. Other proteins displayed strong negative skew of A vs T (-0.06 to -0.31 for C. t aiwanicus and -0.04 to -0.31 for C. ch inensis). The seven protein-coding genes on the H strand show negative GC skew, while the six protein-coding genes on the L strand exhibit significant positive GC skew in the two Cistopus species. In C. t aiwanicus, the maximum and minimum negative GC skew value is -0.6 (ATP8) and -0.14 (CO1), respectively, while the maximum and minimum positive GC skew value is 0.65 (ND6) and 0.37 (Cytb), respectively. In C. ch inensis, the respective corresponding GC skew value is -0.64 (ATP8), -0.17 (CO1), 0.64 (ND4L) and 0.33 (Cytb).

Codon usage patterns

The pattern of codon usage of C. ch inensis and C. t aiwanicus mtDNA was also studied (Table 3). In C. ch inensis, the most frequently used amino acids were Leu (15.08%), Ser (10.41%), Ile (10.18%), Phe (9.04%), and Met (8.96%), while in C. t aiwanicus, the most frequently used amino acids were Leu (15.03%), followed by Ser (10.29%), Ile (9.71%), Phe (9.29%), and Met (8.66%). In the two Cistopus species, the least frequent amino acids were both arginine and glutamine. Individually, C. t aiwanicus employs TTA (leucine) 396 times, while C. ch inensis employs 401 times for protein synthesis. TTA (leucine) is definitely the most frequently used codon not only in the two Cistopus species, but also in other octopods.

Transfer RNA and ribosomal RNA genes

The mt genome of C. t aiwanicus has the complete set of 22 tRNA genes, while that of C. ch inensis has 24 tRNA genes, with additional tRNA-Phe1 and tRNA-Leu3 genes (Figure 1). Most of the tRNAs in the two Cistopus species were identified by the ARWEN program. However, three tRNA genes of C. t aiwanicus were identified by sequence similarity with the previous reported mt genomes of octopods (Table 1). In the two Cistopus species, the tRNA genes vary in length from 60 (tRNA-Phe (GAA) in C. ch inensis) to 77 (tRNA-Ser (TGA) in C. ch inensis) nucleotides with differences in stem and loop sizes of dihydrouridine (D) and TYC loops. The GC content of the tRNA genes ranges from 11.4% to 31.3% in the two Cistopus species. In the C. t aiwanicus mt genome, 8 tRNAs are encoded on the H strand and 14 on the L-strand, while in C. ch inensis, 8 and 16 tRNAs are encoded on the H and L strand respectively. Besides the two additional tRNA genes, the order and orientation of the tRNA gene arrangement pattern of C. ch inensis are identical to that of C. t aiwanicus. In addition, the putative secondary structures of the tRNAs are similar to each other, suggesting similar functions. In the mt genomes of most animals, certain tRNAs generally lacks a DHU arm, instead having a TV-loop or D-loop structure. Previous studies have suggested that the reduction of the tRNA stem was caused by
strong pressure for mt genome minimization [23]. In C. taiwanicus, 19 tRNA genes can be folded into a normal cloverleaf structure, except for tRNA-Ile, tRNA-Gly and tRNA-Glu that lack a DHU arm. In C. chinensis, the DHU arm of tRNA-Phe (GAA) and tRNA-Cys are replaced by a D-loop structure.

In the two Cistopus species, the ribosomal RNA genes of 16S and 12S are located between tRNA-Leu2 and tRNA-Val, and between tRNA-Val and tRNA-Met, respectively. The 16S gene is 1411 bp long in C. taiwanicus and 1332 bp in C. chinensis, with the AT content of 79.38 % and 77.70 %, respectively. In C. taiwanicus, the 12S gene is 990 bp long and the AT content is 80.51 %; while in C. chinensis, it is 995 bp long with an AT content of 80.20 %. Sequence identities in the 16S and 12S genes between C. chinensis and C. taiwanicus are 74.24% and 84.74%, respectively. It seems that the 12S genes are more conserved in the genus Cistopus.

Levels of variability for the protein coding genes

The nucleotide and amino acid sequence similarities for each of the 13 mt proteins in C. chinensis and C. taiwanicus ranged from 73.75%-87.43% and 73.86%-95.61%, respectively. Based on nucleotide similarity, Cytb is the most conserved protein coding gene, while ND3 is the least conserved. According to the amino acid sequences similarity, CO2 is most conserved protein, and ND2 is least conserved. Genes in mt genome may have different evolutionary rates, which might be caused by different selection pressures or the restriction of gene function [24]. The evolutionary rates of mitochondrial genes were also found to diverge differently in cephalopods. To determine the evolutionary rate of each protein, we compared the mt protein-coding and rRNA genes of C. chinensis and C. taiwanicus with that of the published data of Octopodiformes with the same gene order. Based on the nucleotide similarities, the conserved protein coding genes among the six species include CO3 (70.64%-87.18%), CO1 (74.5%-85.52%), CO2 (74.24%-86.75%), Cytb (65%-87.43%) and ND1 (61.5%-83.1%). The least conserved proteins were ND3, ND2 and ATP8, with sequence identity between 44.63%-73.75%, 54.13%-82.66%, and 44.87%-75.64%, respectively. Highest sequence identity of protein coding genes was observed in Cytb (87.43%), CO3 (87.18%) and CO2 (86.75%) gene, which were all between C. chinensis and C. taiwanicus; while the lowest sequence identity was observed in ND6 (41.2%), ND3 (44.63%) and ATP8 (44.87%), between C. chinensis and V. infernalis, between Amphioctopus fangsiao and V. infernalis, and between A. fangsiao and V. infernalis, respectively. The sequence identities of the 16S and 12S genes change greatly between different species, with percent identities being 54.2%-77.94%, and 55.5%-85.9%, respectively. For the 16S gene, the highest nucleotide similarities were observed between C. chinensis and C. taiwanicus, and between C. taiwanicus and Octopus vulgaris, with the percent identities being 77.94% and 70.43%, respectively; the least nucleotide similarities were observed between O. vulgaris and V. infernalis, and between Octopus minor and V. infernalis, with the percent identities being 54.2% and 54.4%, respectively. For 12S genes, the highest nucleotide similarities were observed between C. chinensis and C. taiwanicus, and between C. taiwanicus and Octopus vulgaris, with the percent identities being 77.94% and 70.43%, respectively; the least nucleotide similarities were observed between O. vulgaris and V. infernalis, and between Octopus minor and V. infernalis, with the percent identities being 54.2% and 54.4%, respectively. Combined, these results from pairwise comparisons of nucleotide sequences from the protein-coding genes as well as the rRNA genes suggested that the C. taiwanicus mtDNA most closely resembles its congeneric

| Table 3. Codon usage in 13 protein-coding genes of the Cistopus chinensis (CH) and Cistopus taiwanicus (CW) mitochondrial genomes. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | CH              | TW              | CH              | TW              |
| Phe (CGU)       | TTT             | 306             | Ser             | TCT             | 111             | Tyr             | TAT             | 161             | Cys             | 165             | TGT             | 64              |
| (GAA)           | (UGA)           |                 |                 | (GUU)           |                 | (GUA)           |                 | (GCA)           |                 | (GCA)           |                 | 2               |
| Leu (TG)        | TTA             | 401             | 396             | TCA             | 96              | 101             | Ter             | TAA             | 10              | 9               | Trp             | TGA             | 78              |
| (UAA)           | TG              | 65              | 60              | TCG             | 5               | 4               |                 | TAG             | 3               | 4               | (UCA)           | TGG             | 17              |
| Leu (UG)        | CTT             | 42              | 53              | Pro             | CTT             | 69              | 73              | His             | CAT             | 54              | 64              | Arg             | CGT             | 15              |
| (UAG)           | (UGG)           | 17              | 11              | (UGU)           |                 | CCA             | 13              | 9               | (GUG)           |                 | (UG)            |                 | 1               |
| CTA             | 45              | 47              | CCA             | 34              | 35              | Glh             | CAA             | 51              | 54              |                 |                 | CTA             | 27              |
| CTG             | 2               | 4               | CCG             | 1               | 2               | (UUG)           |                 | (UGC)           | 8               | 7               |                 | (UUC)           |                 | 10              |
| Ile (ATG)       | ATT             | 348             | 344             | Thr             | ACT             | 51              | 67              | Asn             | AAT             | 163             | 154             | Ser             | AGT             | 53              |
| (GUA)           | 38              | 25              | (UGU)           | 17              | 11              | (GUU)           |                 | (GCU)           | 22              | 25              | (GCU)           |                 | 6               |
| Met (ATA)       | 274             | 284             | ACG             | 4               | 9               | (UU)            |                 | 13              | 21              |                 |                 | AGG             | 33              |
| (CAU)           | ATG             | 46              | 45              | ACG             | 4               | 9               | (UU)            | 13              | 21              |                 |                 | AGG             | 33              |
| Val (GGT)       | 87              | 89              | Ala             | GCT             | 53              | 57              | Asp             | GAT             | 69              | 63              |                 |                 | GGT             | 84              |
| (UAC)           | GC              | 6               | 8               | (UGC)           | 14              | 11              | (GUC)           |                 | 5               | 12              | (UCC)           |                 | GGC             | 6               |
| GTA             | 86              | 84              | GCA             | 38              | 43              | Glu             | GAA             | 62              | 70              |                 |                 | GAA             | 91              |
| GTG             | 20              | 29              | GCG             | 6               | 7               | (UUC)           |                 | (UAC)           |                 |                 | (UG)            |                 | 45              |

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species C. chinensis, and V. infernalis is more distant to the other Octopodiformes.

**Sliding window analysis of mt genomes**

Sliding window analysis of the complete nucleotide alignment of six available Octopodiformes mtDNAs provided an indication of nucleotide diversity $\Pi$ within and between mt genes (Figure 2). In the curve, the nucleotide variation within and between mt genes among the aligned Octopodiformes genomes was displayed for any given window of 200 bp and steps of 20 bp, with the $\Pi$ value ranging from 0.069 to 0.401. Coupled with computation of the number of variable positions per unit length of gene, the sliding window showed that the genes with low sequence variability included CO1 (0.345), CO2 (0.339), CO3 (0.356) and Cytb (0.336), while the genes with high sequence variability included ND3 (0.561), ND2 (0.533), ATP8 (0.513), ATP6 (0.498), ND5 (0.467), ND4 (0.473), ND4L (0.5), ND6 (0.506), ND1 (0.418), 16S (0.531) and 12S (0.511). Interestingly, the genes with pronounced peaks and troughs of $\Pi$ appeared to possess higher sequence variability than others, such as ND3, ND2, ATP8 and 16S (Figure 2). Based on these results, it seemed that CO3, CO1, CO2 and Cytb were the most conserved protein-coding genes, and ND3, ND2 and ATP8 were the least conserved ones. The two rRNA genes also showed much high sequence variability. These observations were consistent with the findings from pairwise comparisons made among the nucleotide sequences from the protein-coding genes of Octopodiformes. These results further suggested that there are still a considerable number of alternative genes that could be developed as new genetic markers for phylogenetics and population genetics in octopods.

Current mt genes widely used as molecular targets for PCR assays based approaches for detection of octopods include CO1, CO3 and 16S genes [7,25]. Although relatively easy to amplify routinely, based on pairwise comparison and sliding window analysis of mt genes among the octopodiform mtDNAs, CO1 and CO3 are the slowest evolving and least variable genes. Therefore, more reliable, or at least more informative markers should be considered for future work, especially for the detection involving species with similar phenotypes. From the analysis in the present study, compared with the CO1 and CO3 genes, it seemed that ND1 and ND5 may be more suitable as molecular genetic markers for identification of octopods, due to their relatively higher sequence variability. As shown in sliding window analysis, both ND1 and ND5 genes were found to possess more variable positions per unit length of gene than CO3 and CO1 (Figure 2). Perhaps these markers can be further validated when additional octopods mt genomes become available, especially from the family Octopodidae.

**Phylogenetic analyses**

Many systematic and population genetic studies have been based on genetic markers in the mt genomes at both the nucleotide and amino acid levels [26]. Previous studies have indicated that usage of complete mt sequences for phylogenetic analyses would be more reliable in cephalopods. To better understand the evolution of genome-level features in cephalopod species and assess the phylogenetic position of Cistopus species, phylogenetic relationships among completely sequenced cephalopod species were inferred from concatenated amino acid sequences of the 13 mt protein-coding genes. The phylogenetic relationships of 22
cephalopods based on concatenated amino acid sequence datasets, plus the mt DNA sequence of two *Cistopus* species obtained in the present study, are shown in Figure 3. In the tree, two major clades were recovered within Coleoidea; clade I and clade II form monophyletic groups, respectively. Within clade I, Decapodiformes species was divided into four monophyletic groups, consisting of Oegopsina, Myopsina, Sepiolidae and Sepiina. This result was highly consistent with taxonomic classification based on morphological data. Within clade II, *C. chinensis* and *C. taiwanicus* clustered together with high statistical support, indicating that *C. chinensis* and *C. taiwanicus* have a sister group relationship. These results further confirm the taxonomic classification of the two *Cistopus* species by morphological data analysis.

The family Octopodidae contains numerous undescribed species, and the taxonomy of many species is highly contentious [27]. The identification of octopod species is an international technique problem, because of the lack of informative morphological characters. The only successfully used features include the chitinous beaks, small internal rods in

**Figure 3.** Inferred phylogenetic relationships among the cephalopods based on mitochondrial DNA sequences. The concatenated amino acid sequences of 13 protein-coding genes were analyzed ML, NJ, MP and TNT analysis, using *Nautilus macromphalus* as outgroup. The number at each node is the bootstrap probability of ML analyses. Bootstrap values generated from 1000 replicates for NL, MP and TNT analysis, while 100 replicates for ML analysis.

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the dorsal mantle musculature, chitinous radula teeth, mineralized balance organs and the unique calcareous external and unattached shell in females of the pelagic octopods [28]. The search for new morphological characters that could help classify octopods dates back centuries. There are significant morphological differences between C. chinensis and C. taiwanicus. For example, the former is a small to moderate-sized octopus, reaching ML to around 99.5 mm, and weight to 94.6 g, while the latter is a medium to large-sized octopus, reaching a ML of about 150 mm, and weight of 1200 g. The habitat of C. chinensis is different from that of C. taiwanicus. The former prefers muddy bottom, while the latter prefers rocky substrates. In addition, the size of the germ cells of the two Cistopus species also differs obviously. The common feature of the two Cistopus species is the mucous pouch on the oral surface of the webs. The phylogenetic analyses based on mtDNA sequence indicated a close relationship of the two Cistopus species, indicating the importance of mucous pouches for species identification. The mucous pouches provide valuable morphological features for octopus classification.

The higher-level systematic relationships within Octopodidae remains poorly understood, due to the problems in identifying informative morphological characters. The phylogenetic analyses of mtDNA sequence appear to be a powerful and valuable tool for classification of Octopodidae in the absence of sufficient informative morphological data. In the phylogenetic analyses, the monophyletic group of Octopodidae consisted of five species including two Cistopus species, one Octopus species, one Amphioctopus species and one Callistoctopus species (Figure 3). Interestingly, these four genera of octopods could be successfully clustered into four groups. In the tree, the species C. chinensis and C. taiwanicus combined to form a monophyletic group, while Octopus vulgaris appears as sister to this monophyletic group. The octopod Amphioctopus fangsiao forms a new group with the above monophyletic group, while Octopus minor is supported as sister to the combined group. The Cistopus species and O. vulgaris formed a sister group, indicating a closer relationship between Cistopus and Octopus than to Amphioctopus. The taxonomic status of Octopus minor (Sasaki, 1920) (Cephalopoda: Octopoda) was previously assigned as Octopus or as unclear [29]. Recently, this species was placed in the genus Callistoctopus according to phylogenetic analyses of CO1 and CO3 [30]. Our results indicated that O. minor exhibited relatively distant genetic relationships with the other three octopod genera, providing evidences for the removal of this species from Octopus. However, since the lack of available references from Callistoctopus genus, whether the species Octopus minor (Sasaki, 1920) should be attributed to Callistoctopus or other octopod general remains unknown and still needs further study. The controversy regarding the phylogenetic status of Vampyromorpha was still not well resolved according to our results. In the NJ tree analysis, V. infernalis forms a monophyletic group with Decapodiformes, while in the ML and MP tree analysis, it is supported as a sister group of Octopoda although neither arrangement was strongly supported (Figure 3). Complete mt genomes of cirrate and incirrate octopods may contribute to understanding the position of Vampyromorpha in cephalopods. A representative and dense sampling of Octopodiformes subgroups may contribute to resolving contentious interclass relationships in the future, and is vital for exploring the evolution of especially diverse mitochondrial genomes in octopods [31].

In conclusion, the present study determined the mt genome sequence of C. chinensis and C. taiwanicus, which represent the first sequenced mt genomes of the genus Cistopus. The C. chinensis mt genome exhibits novel mt gene arrangement compared with C. taiwanicus and other octopods. Phylogenetic analyses indicated a close relationship between C. chinensis and C. taiwanicus, further confirming the previous taxonomic classifications. Our results also demonstrated the importance of mucous pouches sets in the webs during octopus identification. Characterization of the two mt genomes has contributed to our understanding of the taxonomic classification of Octopodidae, and provided insights into mt genome evolution, especially gene rearrangements in the family.

Materials and Methods

Ethic Statement

All the specimens used in the experiments were collected and treated ethically. The species of C. chinensis and C. taiwanicus used here are very common in the area of southern China and Taiwan. Therefore, this study did not involve endangered or protected species and no specific permissions were required for collecting samples from these locations or activities.

Sample Origin and PCR Amplification

The C. chinensis and C. taiwanicus female adults were collected from the coastal water of Xiamen and Taiwan, respectively. Muscle tissue was preserved in 75% ethanol and stored at 4°C until used for DNA extraction. Total genomic DNA was isolated from a small portion of the specimen using the Universal Genomic DNA Extraction Kit Ver. 3.0 (TaKaRa, Japan). The entire mt genome was amplified in five overlapping segments according to our previous studies [3]. Briefly, partial sequences of the five conserved genes were firstly amplified by universal primers listed in Table S2. The nucleotide sequences obtained from these five genes were then used to design specific primer sets for long PCR amplification of the entire mt genomes. Five overlapping long PCR fragments covering the entire mt genome of C. chinensis and C. taiwanicus were obtained, respectively. The Long-PCR reaction volume amounted to 50 μl containing 31.5 μl sterile deionized water, 5.0 μl 10×LA PCR Buffer (MgCl2 plus), 8.0 μl dNTPs (2.5 mM each), 1 μl each primer (25 pmol/ml), 0.5 μl LA Taq DNA polymerase (Takara) and 3 μl DNA template. Long-PCR cycling conditions used were 94°C for 5 min (initial denaturation), then 94°C for 10 s (denaturation), 52°C for 1 min (annealing), and 68°C for 5 min (extension) for 32 cycles, followed by a final extension at 68°C for 10 min. All amplifications were done on a gradient thermocycler. The 5 long-PCR fragments were sequenced using a primer-walking strategy. The complete nucleotide sequence has been
submitted to GenBank (accession number KF017605 for *C. taiwanicus* and KF017606 for *C. chinensis*).

**Gene annotation and sequence analysis**

Sequences were assembled manually and aligned against the complete mt genome sequence of three Octopoda species using the computer program Clustal W to identify gene boundaries. Protein coding genes were analyzed by ORF Finder using the invertebrate mitochondrial code. Protein genes were identified by comparing predicted amino acid sequences with amino acid sequences of previously identified cephalopods. Ribosomal RNA (rRNA) genes were identified by nucleotide sequence homologies to RNA sequences of the sequenced octopods. Transfer RNA (tRNA) genes were identified in sequences between protein and rRNA genes by their ability to fold into the cloverleaf structures characteristic of mt-tRNA genes of other metazoans, and from the trinucleotide in the anticodon position of these structures either using the ARWEN program or Blast search with the other cephalopods [32]. Base composition and codon usage were calculated in DNASTar software. For sliding window analyses, the complete nucleotide sequences of mtDNAs for six Octopodiformes were firstly aligned using Clustal W. Subsequently, the complete alignment was used to accomplish sliding window analyses with the DnaSP ver.5.10 software package [33]. A sliding window of 200 bp and steps of 20 bp were used to estimate nucleotide diversity Pi for the complete alignment. Nucleotide diversity for the complete alignment was plotted against midpoint positions of each window, and gene boundaries were indicated.

**Phylogenetic analyses**

Phylogenetic relationships among the 22 sequenced cephalopod species, plus the mt DNA sequence of the two *Cistopus* obtained in the present study was reconstructed based on amino acid sequences of 13 protein-coding genes using *Nautilus macromphalus* as the outgroup. The 13 protein coding genes of all sequenced cephalopods were downloaded from GenBank as amino acid sequences and checked for translational accuracy. Translations of the 13 protein coding genes of *C. chinensis* and *C. tawianicus* were analyzed by ORF Finder. Firstly, each gene was aligned using MUSCLE with default settings [34]. Areas of dubious alignment were isolated using Gblocks Server with a less stringent selection (allow for smaller final blocks and 50% gap positions) and excluded from the analysis [35]. Then, the 13 separate amino acid sequence alignments were concatenated to a single multi-sequence alignment, which consisted of 3638 amino acid sites. Three different inference methods, namely neighbor joining (NJ), maximum likelihood (ML) and maximum parsimony (MP), were used for phylogenetic reconstructions. The NJ and MP phylogenetic reconstructions were conducted with MEGA5 under the model of Jones-Taylor-Thornton (JTT) and Subtree-Pruning-Regrafting (SPR), respectively [36]. ML analysis was performed by PHYML 3.0 [37] under the MtArt+I+G+F model amino acid substitution selected with ProtTest program based on the Akaike information criterion (AIC) [38]. Tree searching used a combination of subtree pruning and regrafting (SPR) and NNI on ten random starting trees. Branch supports were evaluated by bootstrapping analysis of 1000 replicates for NJ and MP trees, and 100 replicates for the ML tree. The program TNT (Tree analysis using New Technology) was also applied to construct the phylogenetic tree [39].

**Supporting Information**

**Table S1.** The nucleotide composition and skew analysis of the mitochondrial 13 protein coding genes in *Cistopus chinensis* (CH) and *Cistopus tawianicus* (CW).

**Table S2.** Primers used for the mtDNA amplification of *Cistopus chinensis* and *Cistopus tawianicus*.

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**Author Contributions**

Conceived and designed the experiments: RC XZ QL. Performed the experiments: RC XZ YM. Analyzed the data: RC XZ YM QL. Contributed reagents/materials/analysis tools: RC XZ YM QL. MANUSCRIPT REVIEWS: RC XZ YM QL.

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