

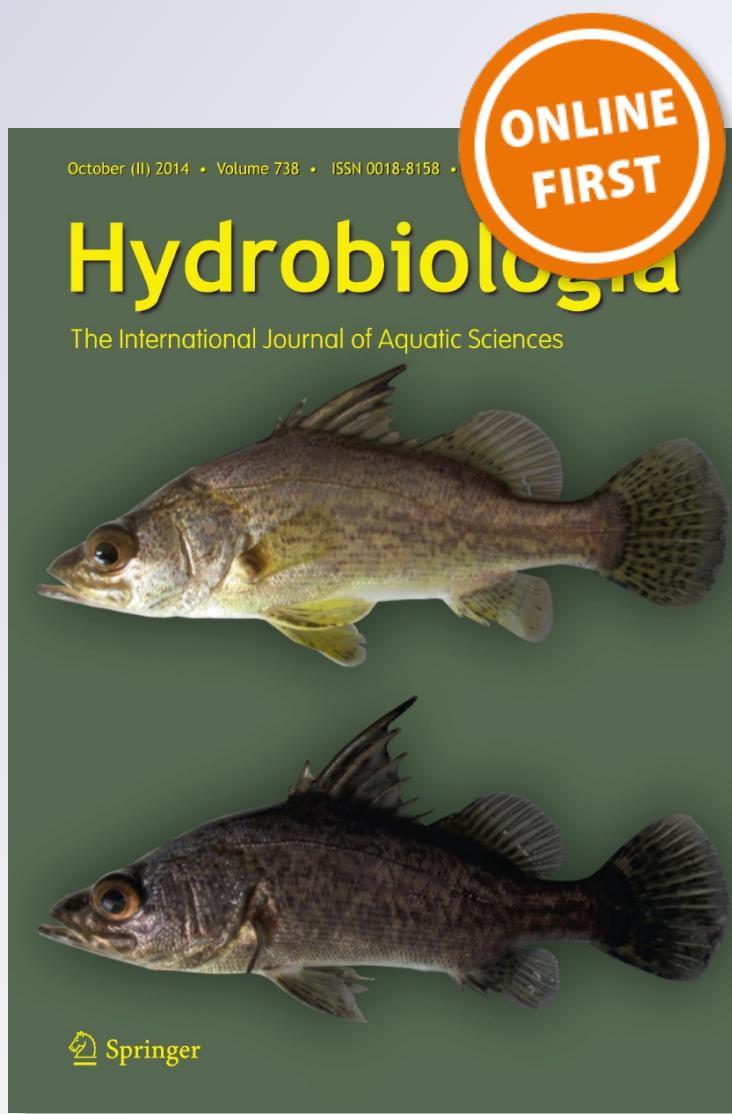
Population genetic patterns of the copepod Calanus sinicus in the northwest Pacific

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Population genetic patterns of the copepod *Calanus sinicus* in the northwest Pacific

N. V. Schizas · H.-U. Dahms · M. Ricaurte ·
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Abstract The calanoid copepod *Calanus sinicus* is present throughout the year in the Bohai Sea, and is transported by the China Coastal Current southwards during winter. The seasonal movement of *C. sinicus* throughout its distribution should result in the genetic homogenization of the species. We tested the hypothesis of the absence of population structure in *C. sinicus* by analyzing the nuclear ribosomal region ITS-1 ($n = 197$, 303 bp) and the mitochondrial gene, cytochrome b apoenzyme (cytb) of *C. sinicus* ($n = 155$, 318 bp) from locations in Taiwan, S. Korea, and

Okinawa, Japan. Twenty-eight haplotypes were identified from the ITS-1 gene and the most numerous haplotype ($n = 160$) was found everywhere except in S. Korea. Nine haplotypes were identified from the cytb gene and the two most numerous haplotypes ($n = 140$) were widespread. An excess of singletons characterized the haplotype networks of ITS-1 and cytb genes. Pairwise F_{ST} comparisons among samples suggested that samples from S. Korea, S. Taiwan Strait, and Taipower Plant were significantly different from all other samples suggesting a high degree of structured populations in *C. sinicus*, for the ITS-1 gene. In the contrary, no population structure was detected with the cytb gene. Pairwise F_{ST} comparisons among samples collected at different water depths in Turtle Island suggest that populations at depth may contribute to the genetic differentiation of *C. sinicus*. The two genes yielded conflicting genetic signals as the aforementioned geographic and depth-related population demarcations were observed only with the ITS-1 gene.

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Introduction

The composition of zooplankton communities has a long tradition for being used as an indicator of water movements (Peterson & Keister, 2003, Hwang & Wong, 2005, Hwang et al. 2006). That copepods can provide suitable indicators for water mass movements

that are otherwise characterized by different temperatures and salinities, is well established (Hsieh et al., 2004, Hwang et al., 2007, Dahms & Hwang, 2010, Tseng et al., 2011).

Complex water exchange affects the composition and abundance of oceanic biota around the island of Taiwan. This holds true for both the microzooplankton (Vandromme et al., 2010; Chang et al., 2011) and the mesozooplankton, (Liao et al., 1999; Tseng et al., 2008d, 2013a, b, c; Chou et al., 2012; Hsiao et al., 2013). It has been estimated that around 10% of the world's marine species can be found in waters around Taiwan (Shao, 1998), making the marine biota in the waters around the island of Taiwan highly diverse. Several authors explain this high biodiversity as being caused by the convergence of different large water masses (Jan et al., 2002; Hwang et al., 2006; Hwang et al., 2007; Hwang et al., 2010b). Temperate species that are transferred from the north and tropical species that are carried from the south via the Taiwan Strait influence the composition of the mesozooplankton communities of Taiwanese waters, which are dominated by copepods (Hwang & Wong, 2005; Hwang et al., 2004, 2006; Dur et al., 2007; Tseng et al., 2008a, b, c, d; Lan et al., 2009; Hwang et al., 2009, 2010a).

The calanoid copepod *Calanus sinicus* belongs to the mesozooplankton fauna of Taiwan, is highly abundant and is an important component of the continental shelf food web in the northwest Pacific Ocean, by providing links between primary productivity and the larvae and juveniles of fishes (Uye, 2000). A study by Hwang & Wong (2005) indicated that *C. sinicus* was transported by the China Coastal Current (CCC) toward northern Taiwan as far as south of Hong Kong. *Calanus sinicus* was never found in summer samples from the coastal areas in Hong Kong waters but was frequently recorded with a high occurrence rate and relative abundance in the period from October to April (Hwang & Wong, 2005; Hwang et al., 2006). *C. sinicus* is not an eurythermal species and temperature seems to be an important factor regulating its distribution in the shelf ecosystem of the northwest Pacific Ocean (Uye, 2000; Hwang & Wong, 2005; Hwang et al., 2006). Chen (1992) reported that the temperature range of *C. sinicus* was 5–23°C with an upper thermal limit at 26–27°C (Wang et al., 2003) with temperatures >23°C being stressful to *C. sinicus* (Uye, 1988).

In northern Taiwan, long-term studies of copepod successions indicated that *Calanus sinicus* is an

important indicator species for cold water masses from the Bohai Sea and the Yellow Sea (Hwang et al., 2004, 2006; Dur et al., 2007). Hwang et al. (2004) described *C. sinicus* as rare in samples from July to September and dramatically increased its density and occurrence ratio in October. As yet, however, it is not known when *C. sinicus* reaches the coastal areas of northern Taiwan from the north.

Contrary to previous studies (Hwang et al., 2004, 2006; Hwang & Wong, 2005; Dur et al., 2007; Tseng et al., 2008b) that indicated *C. sinicus* to be most likely transported by the cold CCC water, *C. sinicus* was recorded in the southern East China Sea (ECS), close to the northern Taiwanese waters, even during the northeast monsoon transition period. Hsieh et al. (2004) claimed that *C. sinicus* was still located north of the Taiwan Strait when the northeast monsoon was prevailing. Liao et al. (2006) found *C. sinicus* during August not only in sampling stations influenced by the Taiwan Strait warm current but also in stations influenced by the cold-core upwelling of the southeastern ECS. Liao et al. (2006) inferred that *C. sinicus* in fact comes from the waters of the northern Taiwan Strait and later appears at the surface due to upwelling. *C. sinicus*, therefore, might seek refuge to deeper colder waters in the northern Taiwan Strait during the summer period, and might return to the surface waters via cold upwelling from deeper areas or via vertical migration. Reservoir populations of *C. sinicus* present in the colder, deeper upwelling waters of northern Taiwan may influence the population genetic structure of the species through spatial isolation.

It is obvious from the literature that the distribution and migratory patterns of *C. sinicus* requires further refinement. Genetic techniques offer us the possibility to infer indirectly connectivity patterns and relationships between different populations. We will test for the first time, if the seasonal transportation of *C. sinicus* results in genetic panmixia and whether there are reservoirs of genetically distinct populations of *C. sinicus* in deeper waters of the northern Taiwan Strait. These hypotheses will be assessed with the nuclear ribosomal gene Internal Transcribed Spacer 1 (ITS-1) and the mitochondrial gene cytochrome b apoenzyme (cytb). The cytb and ITS-1 genes are used frequently in population level studies in harpacticoid and calanoid copepods (e.g. cytb: Schizas et al. 1999, 2002, Staton et al. 2003, Papadopoulos et al. 2005; ITS-1: Schizas et al. 1999, Rocha-Olivares et al. 2001, Ki

Table 1 Genetic diversity and summary statistics of *Calanus sinicus* based on ITS-1 and cytb sequences

Localities		<i>N</i>	<i>H</i>	π	θ_s	<i>D</i>	<i>F_s</i>	Latitude (N)	Longitude (E)	WD
Okinawa, Japan	ITS-1	22	3	0.00030	0.27432	-1.16240	-3.22865*	26°18'	127°33'	0
S. Korea	ITS-1	9	9	0.05078	18.0289	-0.95814	-1.95370	37°17'	126°22'	0
	cytb	17	2	0.001395	0.295794	0.94981	1.06874			
Taipower plant	ITS-1	11	4	0.00352	1.36567	-0.83418	-0.62653	25°11'	121°22'	0
	cytb	13	6	0.003986	1.611234	-0.78512	-2.43533*			
S. Taiwan Strait	ITS-1	19	9	0.01064	5.72228	-1.73588*	-1.75496	23°07'	119°52'	0
	cytb	33	3	0.00169	0.504840	0.11561	0.19123	24°30'	122°21'	
Turtle Is. 8 m	ITS-1	8	6	0.012831	5.01377	-1.20422	-1.05906	24°30'	122°21'	8
	cytb	17	4	0.001957	0.887383	-0.86089	-1.25588			
Turtle Is. 40 m	ITS-1	18	2	0.000369	0.29074	-1.16467	-0.79427	24°30'	122°21'	40
	cytb	15	3	0.001931	0.615089	-0.02381	-0.06433			
Turtle Is. 45 m	ITS-1	25	1	0	0	0	0	24°30'	122°21'	45
	cytb	13	4	0.002682	0.966741	-0.39439	-0.87399			
Turtle Is. 80 m	ITS-1	56	2	0.000238	0.43539	-1.45172*	-0.96027	24°30'	122°21'	80
	cytb	22	3	0.001867	0.548643	0.16560	0.17064			
Turtle Is. 180 m	ITS-1	29	2	0.008195	8.40299	-2.66913*	6.91414	24°30'	122°21'	180
	cytb	28	4	0.001755	0.513946	0.15861	-3.67854*			

Significant values ($P < 0.05$) are represented by an asterisk. All locations are in Taiwan or near Taiwan except those from Okinawa, Japan and S. Korea

GC Geographic coordinates, WD Water depth (m), *N* number of samples, *H* number of haplotypes per location, π nucleotide diversity, θ_s Watterson's Theta, *D* Tajima's *D*, *F_s* Fu's *F_s*

et al. 2009) because they harbor sufficient amounts of genetic diversity. The combined use of markers from different genomes is preferred for phylogeographic studies because they provide independent views of the organismal history (Avise 1994).

Materials and methods

Field sampling, identification, and enumeration

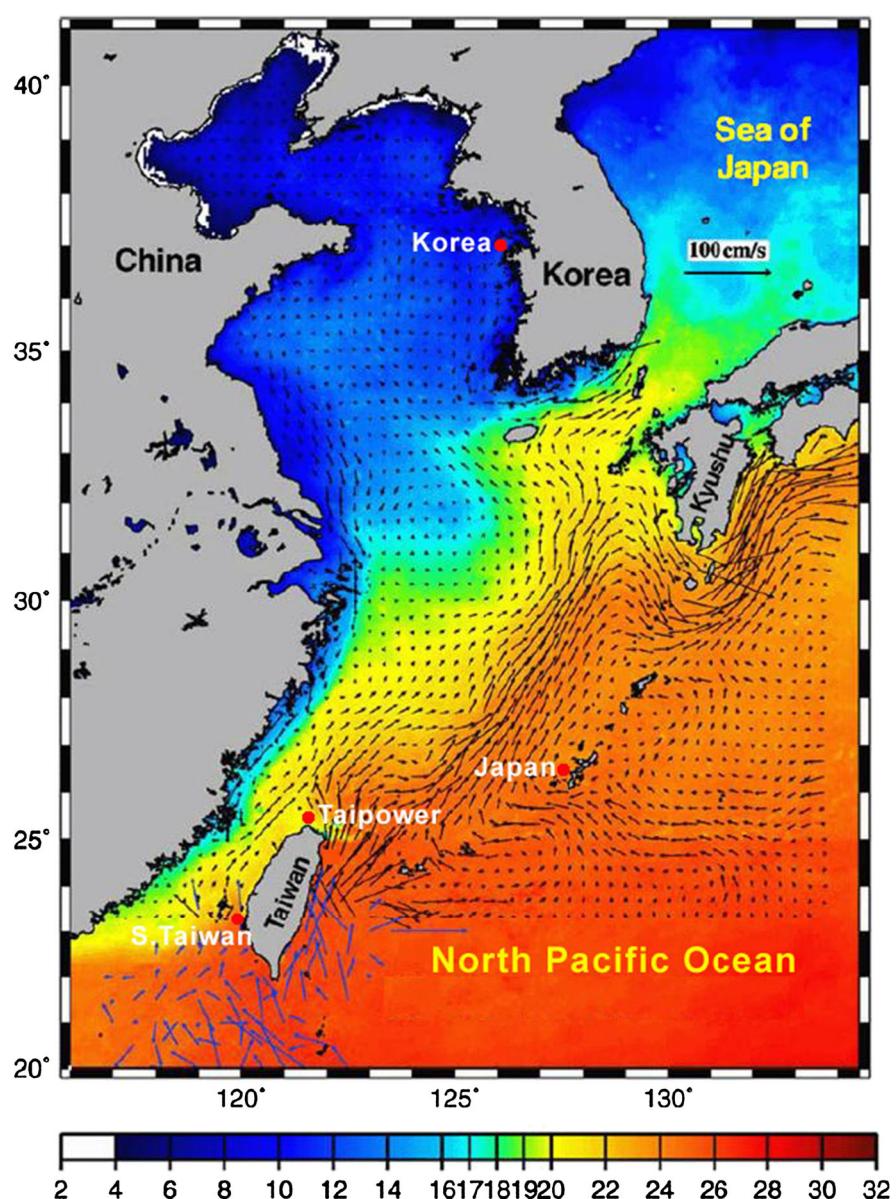
For the present study, zooplankton was sampled at various locations in the West Pacific Ocean (see Table 1; Fig. 1). Zooplankton samples were collected by using oblique tows with a standard North Pacific zooplankton net (mouth diameter 45 cm and a mesh size of 333 μm) at surface and specific depths at stations. A Hydrobios (Kiel, Germany) flowmeter was strapped in the center of the net opening for later estimation of the seawater volume filtered. After retrieval from the ocean, the zooplankton samples were immediately preserved in >95% ethanol solution for further enumeration and identification. Prior to plankton collection, a conductivity-temperature-depth

(CTD) instrument was used to obtain information about temperature and salinity from the area where zooplankton tows were subsequently taken.

In the laboratory, samples were split by a Folsom splitter until the subsample contained fewer than 500 specimens. Zooplankton and copepods were sorted and identified at the species level using the identification keys of Chen & Zhang (1965), Chen et al. (1974) and Chihara & Murano (1997). Additional original taxonomic papers were used for species identification, when required.

A portion of the nuclear ribosomal region ITS-1 (303 bp) and the mitochondrial cytb gene (318 bp) was sequenced from 175 to 155 specimens, respectively, of *C. sinicus*, from 3 locations around Taiwan, including Turtle Island (= Kueishan Tao), and one location from South Korea (Table 1; Fig. 1). We augmented our data set by including 22 ITS-1 sequences of *C. sinicus* from Okinawa, Japan (Nonomura et al. 2008; Genbank Accession Numbers AB297697-AB297701, AB332345-AB332361). All copepods were collected from surface samples except those from Turtle Island, which were collected from 8, 40, 45, 80, and 180 m depth (Table 1). The copepods

Fig. 1 Map of the study area indicating sampling locations. Different colors indicate mean annual surface temperatures (in °C)



from Japan were either collected from 0 to 50 m or between 200 and 400 m and they were all copepodites (Nonomura et al., 2008). The copepods *C. pacificus* (GenBank AB297704) and *C. jashnovi* (GenBank AB297703) were used as outgroups for the phylogenetic reconstruction of the ITS-1 tree.

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted using DNA from individual adult copepods with of DNAzol® Direct.

Specifically, an adult copepod was added to a mix of 10 µl of DNAzol® Direct and the tissue was lysed by a combination of mechanical break up by a pestle and incubation for 15 min at room temperature. The lysate was briefly vortexed and a 2-µl aliquot was used as a template directly into 20 µl of PCR mix.

For the amplification of the ITS-1 region, we used the forward 18S-F (5'-GGT ACC CTT TGT ACA CAC CGC CCG TCG CT-3') and reverse 28S-R (5'-GCT TTG GGC TGC AGT CCC AAG CAA CCC GAC TC-3') primers. For the amplification of the cytb region, we used the forward 151F (5'-TGT GGR GCN

ACY GTW ATY ACT AA-3') and 270R (5'-AAN AGG AAR TAY CAY TCN GGY TG-3') primers (Merritt et al., 1998). PCR assays were performed using the BioMix (Bioline, UK). Each reaction mix contained 50 ng of DNA (2 μ l), 10 μ l BioMix Red, 7 μ l dH₂O, and 0.5 μ l each of 25 mM forward and reverse primers. The targeted gene area was amplified in a Thermal Cycler (Mastercycler Gradient, Eppendorf) for 35 cycles. The PCR program for both genes consisted of an initial denaturation step of 3 min at 95°C, followed by 35 amplification cycles consisting of 95°C for 15 s, 1 min annealing at 45, and 72°C extension for 1 min, ending with a final 7 min extension at 72°C. Originally, we tried to amplify the cytochrome oxidase subunit I with the Folmer primers (Folmer et al., 1994) but we had very little or no yield during our amplifications. For the cyt b gene, we had suboptimal amplifications but about 70% of the specimens yielded amplicon products for Sanger sequencing. The amplified products were purified with the Exosap procedure. All successful PCR products were processed for sequencing using the Big Dye 3.1 Terminator Cycle Sequencing Kit and the forward 18S and 151F primer. The ethanol-precipitated products were loaded into an ABI 3730xl 96-capillary Genetic Analyzer at the High Throughput Genomics Center, Seattle, Washington. All sequences and final alignments have been submitted to GenBank (Accession Numbers KM378741–KM379070) and TreeBASE.

Data analysis

DNA trace files were imported in CodonCode Aligner v.3.7.1.1 for visualization, quality assessment, contig assembly, base calling, and end trimming. Sequences were aligned with the CodonCode Aligner's built-in alignment algorithm using the default parameters. Aligned sequences were imported to DnaSP v.5.10 (Librado & Rozas, 2009) and Arlequin v.3.5 (Excoffier & Lischer, 2010) for general statistical analysis. The DNA neutrality statistic of Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) was computed in Arlequin to test for deviation from the neutral model of molecular evolution (Kimura, 1968). Since this is the first population genetic study of *C. sinicus* we are also interested in the demography of the species. We used these statistics to test if population sizes are increasing (if D \ll 0, or a negative Fu's Fs) or decreasing from a recent bottleneck (if D \gg 0). A negative D is also

indicative of purifying selection and a positive D is indicative of overdominant selection at this locus. The most appropriate model of DNA substitution was determined by the Bayesian Information Criterion in jModelTest (Posada, 2008), after likelihood calculations were carried out in PhyML (Guindon & Gascuel, 2003), and was applied to MCMC-based Bayesian Inference (BI). Population differentiation and the geographical pattern of variation were examined by hierarchical analyses of molecular variance (AMOVA) (Excoffier & Lischer, 2010) and we used pairwise comparisons to decompose variation into components within populations, between pairs of populations within regions, and between pairs of populations between regions. We use the term region to denote all sampling locations within the Taiwanese waters. Samples collected from different depths in Turtle Island were treated as distinct populations throughout the analysis. Fixation indices or Φ -statistics represent the proportion of each component of variation, relative to the total variation (Weir et al., 1984). Φ_{ST} represents the proportion of variation among populations, Φ_{SC} represents the proportion of variation among regions, and Φ_{CT} represents the proportion of variation among populations within regions. The significance of Φ -statistics was assessed by 10,000 permutations of groups and haplotypes. Insignificant/very small values of Φ -statistics (close to 0) would indicate extensive gene flow among populations and no population structure. Significant/larger values of Φ -statistics would indicate reductions or absence of gene flow among populations. Pairwise comparisons among populations were used to identify which pairs of populations were significantly different from each other.

The relationship between the genealogy of haplotypes and their distribution was depicted in haplotype networks inferred in Network v.4.6 (Fluxus-engineering Comp.) with the full median joining algorithm (Bandelt et al., 1999) and illustrated with Network Publisher v.2.0 (Fluxus-engineering Comp.) after ITS-1 and cyt b sequences were imported to DNA Alignment v.1.3 (Fluxus-engineering Comp.) for formatting without any changes to the original alignment. ITS-1 and cyt b sequences were imported to MrBayes v.3.2.1 (Ayres et al., 2012; Ronquist et al., 2012) to construct the Bayesian genealogies. The conditions for the Bayesian analysis were as follows: four independent runs were conducted with 3,000,000 generations,

starting with random trees, employing four simultaneous chains, and sampling every 100 generations and discarding the first 10% of the sampled trees as burn-in. Clade support for BI was evaluated with posterior probabilities (pP). To check the performance of MrBayes output, we used Tracer version 1.5 (Rambaut & Drummond, 2007) to summarize the posterior distributions of trees and parameter values for an adequate convergence and mixing. Adequate sampling of the posterior probability distribution was verified by the Potential Scale Reduction Factor, which was close to 1.0. Network graphs provide an alternative way of depicting phylogeographic data especially if there are many different but equally most parsimonious genealogies (Mardulyn, 2012) that offer information about possible correlations of sampling localities (=geography) with specific haplotypes (molecular data). A combined approach with phylogenetic trees is suggested though, because each method may yield a different set of parsimony connections, maximizing the chances to identify the most parsimonious solution of that particular DNA dataset (Mardulyn, 2012).

Results

The haplotype network analysis of *C. sinicus* identified 28 haplotypes from 197 sequences of the complete ITS-1 gene (Fig. 2) and nine haplotypes from 155 partial cytb sequences (Fig. 3). The most numerically predominant ITS-1 haplotype ($n = 160$) was found in all sampling locations, except in S. Korea (Fig. 2). The second most dominant haplotype ($n = 10$) was equally represented in the S. Taiwan Strait and at Taipower Plant, Taiwan. The rest of the haplotypes, except one, are represented by one sequence (i.e. singletons) (Fig. 3). The cytb haplotype network was characterized by two numerically predominant haplotypes ($n = 99$ and $n = 41$) found in all sampled locations. The third most abundant haplotype ($n = 9$) was not found in S. Korea (Fig. 3). Neither geographic nor strong depth partition became evident from the network analysis of either gene.

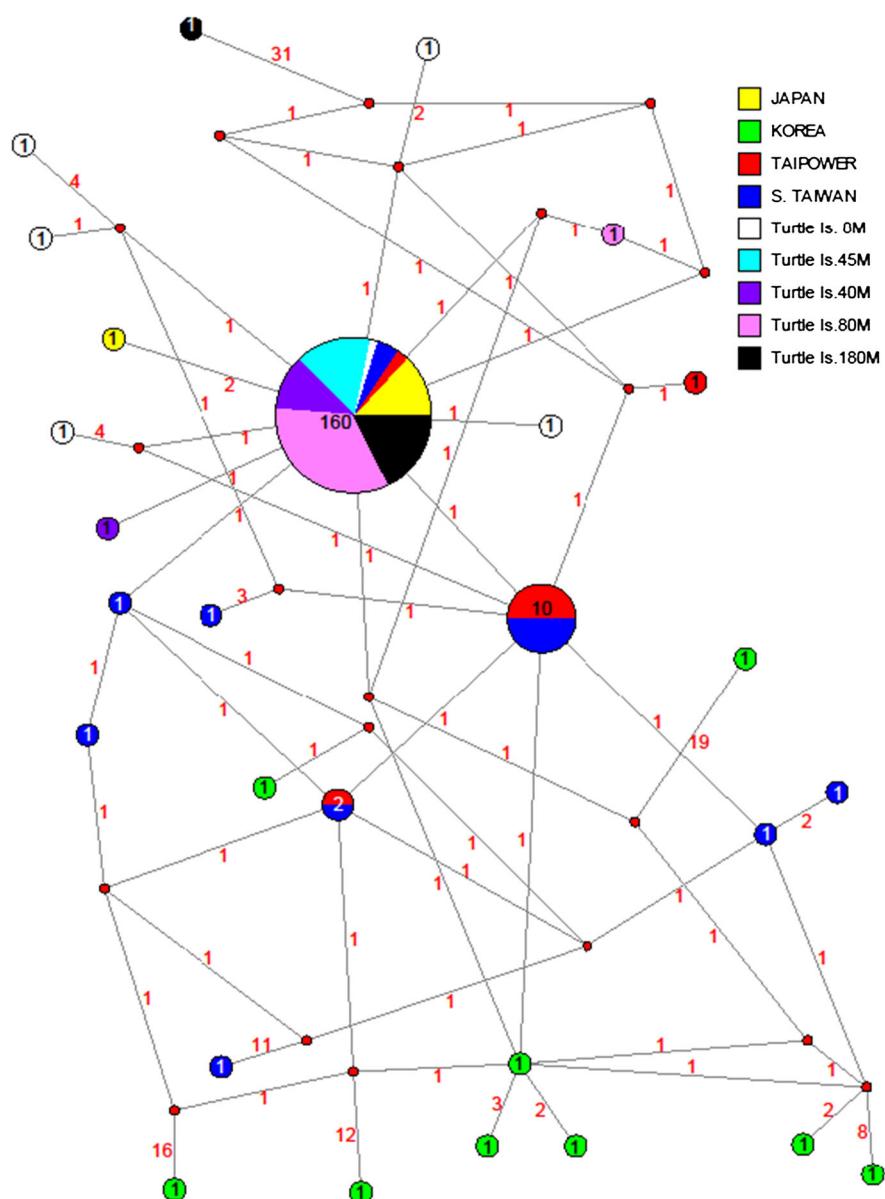
For ITS-1, the nucleotide diversity indices π and θ indicated high variability in the amount of harbored diversity in each sampled location. The highest values of π (0.05078) and θ (18.02891) were reported from S. Korea and the shallowest sample (8 m) from Turtle

Island ($\pi = 0.012831$, $\theta = 5.013774$). The lowest values of π and θ were observed in the 45-m depth sample from Turtle Island where no genetic variation was observed among the 25 studied copepods (Table 1). For the cytb gene, the nucleotide diversity was much lower than that of ITS-1 in S. Korea, S. Taiwan Strait, and 8 m and 180 m depth samples from Turtle Island (Table 1). There are no consistent patterns of levels of nucleotide diversity indices in most locations, for example, in S. Korea, ITS-1 displays order(s) of magnitude higher levels of π and θ than cytb, whereas in Turtle Is. 45 m, exactly the opposite pattern is observed.

Analysis of molecular variance

For the ITS-1 region, population comparisons revealed significant population differentiation ($\Phi_{ST} = 0.302$, $P = 0.000$), non-significant differentiation among populations within regions ($\Phi_{SC} = 0.005$, $P = 0.02$), and significant differentiation among regions ($\Phi_{CT} = 0.298$, $P = 0.000$). AMOVA simulations revealed that the most significant component of variation is found within populations (69.79%; Table 2). Significant differences were also found among regions (29.85%; Table 2). Pairwise comparisons among all samples revealed significant differentiation, most notably those from Turtle Island from all depths, which were significantly different from those of S. Korea, Japan and Taipower Plant (Table 4). *C. sinicus* from Japan was not significantly different against the deeper *C. sinicus* from Turtle Island. Interestingly, the Japan samples were significantly different from all other samples including the shallow samples (8 m) from Turtle Island. The S. Korea, S. Taiwan Strait, and Taipower Plant (except one comparison) samples were significantly different from all other samples (Table 4). With the cytb sequences, in contrary to the results of ITS-1, the hierarchical comparisons in AMOVA indicated the absence of genetic population structure ($\Phi_{ST} = -0.01355$, $P = 0.67911$) (Table 3). Negative values of Φ_{ST} and the other Fixation Indices (Table 3) are regarded as not significantly different than 0. Likewise, there were no significant pairwise comparisons among populations with cytb, not even among the most distant samples (Table 4). We had no samples from Okinawa, Japan so comparisons with this location using the cytb gene were not possible.

Fig. 2 Median-joining network of *Calanus sinicus* based on ITS-1 sequences. The size of circles is proportional to the number of specimens sharing the same haplotype, indicated by the numbers inside the circles. The geographical origin of the sequences making up a particular haplotype is color-coded. The small red circles represent the median vectors. Numbers on connecting lines indicate the number of mutations between haplotypes and internal nodes. *Taipower* Taipower Plant, Taiwan, S. Taiwan South Taiwan Strait, Taiwan



Phylogenetic analysis

The TPM2 + Γ and the HKY model of DNA substitution were selected by the Bayesian Information Criterion (BIC) for the ITS-1 and cytb, respectively. These models were used as priors in BI in MrBayes and in all estimations of population parameters in Arlequin. The specific attributes of the model for ITS-1 were as follows: equal base frequencies, nst = 6, rmat = (a→c:0.2566, a→g: 0.9386, a→t: 0.2566, c→g:1.0000, c→t: 0.9386, g→t: 1.0000), Γ

distribution $\alpha = 0.4340$, ncat = 4, probability of invariant sites = 0. For the cytb gene, the model attributes were the following: nst = 2, base = (0.2313 0.2341 0.1751), ti/tv = 7.0156, rates = equal, probability of invariant sites = 0. There is limited phylogeographic signal in the ITS-1 tree as no major clades identifying distinct populations were observed (Fig. 4). The Korean population is characterized by high diversity as depicted by the long branches of the ITS-1 tree (Fig. 4). Similarly, the Bayesian cytb tree is largely unresolved (figure not shown).

Fig. 3 Median-joining network of *Calanus sinicus* haplotypes based on cytb sequences. The number of specimens sharing the same haplotype is indicated by the number inside the circles. The geographical origin of the sequences making up a particular haplotype is color-coded. Numbers on connecting lines indicate the number of mutations between haplotypes and internal nodes. Taipower Taipower Plant, Taiwan

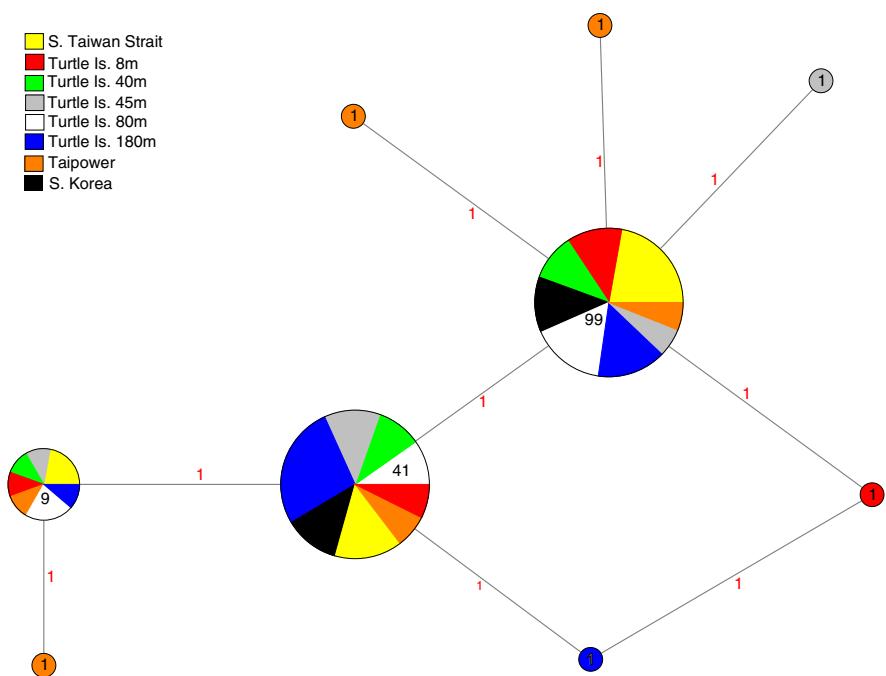


Table 2 Analysis of molecular variance (AMOVA) of *Calanus sinicus* based on ITS-1 sequences

Source of variation	Sum of squares	Variance components	% of variation	Fixation indices
Among regions	34.898	0.32340	29.84561	$\Phi_{CT} = 0.29846$
Among populations within regions	3.411	0.00388	0.35832	$\Phi_{SC} = 0.00511$
Within populations	142.157	0.75629	69.79607	$\Phi_{ST} = 0.30204$
Total	180.467	1.08357		

Total population differentiation using maximum likelihood DNA sequence pairwise distances (Tamura-Nei correction)

Table 3 Analysis of molecular variance (AMOVA) of *Calanus sinicus* based on cytb sequences

Source of variation	Sum of squares	Variance components	% of variation	Fixation indices
Among regions	0.673	-0.00187	-0.57641	$\Phi_{CT} = -0.00576$
Among populations within regions	1.129	-0.00252	0.77872	$\Phi_{SC} = -0.00774$
Within populations	47.934	0.32849	101.35513	$\Phi_{ST} = -0.01355$
Total	49.736	0.32410		

Discussion

The level of genetic differentiation in space and time varies among species and indicates the presence of metapopulations (Rynearson et al., 2009), but there is probably no standard model for population structure

among marine zooplankton species. The dispersal potential of zooplankters is high in the absence of obvious barriers resulting in the absence of population structure (Provan et al., 2008). However, oceanographic features such as gyres may lead to population subdivision (Bucklin et al., 2000; Goetze, 2005; Unal & Bucklin, 2010).

Table 4 Pairwise F_{ST} comparisons of *Calanus sinicus* based on ITS-1 and cytb (below) sequences

	Japan	S.Korea	Taipower	S.Taiwan	Turtle 8 m	Turtle 45 m	Turtle 40 m	Turtle 80 m
S. Korea	0.39128							
	N/A							
Taipower	0.50269	0.18943						
	N/A		-0.00545					
S. Taiwan	0.18158	0.18817		-0.01862				
	N/A		-0.03708		-0.00266			
Turtle 8 m	0.18925	0.17056	0.16111	0.08624				
	N/A		-0.04091		-0.00912	-0.03958		
Turtle 45 m	0.00543	0.42426	0.56950	0.20271	0.22541			
	N/A		-0.00324		-0.04523	0.01465	0.01653	
Turtle 40 m	0.00127	0.35185	0.46516	0.15876	0.15470		0.01885	
	N/A		-0.05395		-0.04293	-0.04390	-0.04315	-0.04249
Turtle 80 m	0.00362	0.59642	0.65330	0.36811	0.31273		-0.01637	0.00930
	N/A		-0.03691		-0.02066	-0.03979	-0.04420	-0.00041
Turtle 180 m	-0.00851	0.34006	0.13106	0.10440	0.04734		-0.00525	-0.01614
	N/A		0.02484		-0.01536	0.04357	0.04728	-0.04996
							-0.01606	0.03065

Genetic distances among populations were corrected by the most complex nucleotide model of substitution (Tamura & Nei, 1993) available in Arlequin. Bold and underlined values denote significant values at the $\alpha = 0.05$

Japan Okinawa, Japan, Taipower Taipower Plant, Taiwan, Turtle Turtle Island, Taiwan

The annual movement of *C. sinicus* by the CCC from the north toward northern Taiwan as far as south of Hong Kong (Hwang & Wong, 2005) would preclude population differentiation. Since only a few migratory breeding individuals per generation are sufficient to create genetic panmixia, the presence of population structure (with the ITS-1 gene) in *C. sinicus* was unexpected. Based on the ITS-1 gene, AMOVA results and pairwise F_{ST} comparisons between populations (Tables 2, 4), copepods from Korea, from the S. Taiwan Strait and Taipower Plant were significantly different from the other samples suggesting the presence of population structure in *C. sinicus*. The second most dominant ITS-1 haplotype ($n = 10$) of *C. sinicus* was present in two locations in Taiwan, only. In contrast, these genetic patterns were not observed in the mitochondrial cytb sequences. For example, the two most dominant haplotypes of cytb (144 out of 155 sequences, in total) were found in all sampling locations (Fig. 3) and no locations were genetically distinct. Low sample size in some locations may have hindered respective conclusions.

We used haplotype networks to depict the population genetic structure of *C. sinicus* (Figs. 2, 3). The ITS-1 haplotype network of the present study is

characterized by a high number of singletons, which is not unusual for marine species with large effective population sizes such as the widespread *Calanus sinicus*. Crandall & Templeton (1993) argued that singletons are more often connected to non-singletons and are more likely connected to the haplotypes in the same population, an observation consistent with our haplotype networks (Figs. 2, 3). However, the haplotype diversity of *C. sinicus* per sampled location did not indicate a consistent geographic pattern of diversity with either gene. The samples of S. Taiwan Strait harbored one of the highest haplotypic diversities (after S. Korea and Turtle Is. 8 m). The expectation from a northern origin for the southern populations of *C. sinicus* is that higher amounts of genetic diversity would be observed in samples from higher latitudes. While S. Korea samples do harbor more haplotypic diversity, the sampling size is small. A higher sequencing effort and more samples from northern latitudes (e.g., Bohai Sea and the Yellow Sea) would be essential to test if higher genetic diversity is encountered at the northern populations.

For the ITS-1 data, depth appears to be a significant factor influencing genetic differentiation of *C. sinicus* in Turtle Island, but not in Okinawa, Japan. *Calanus*

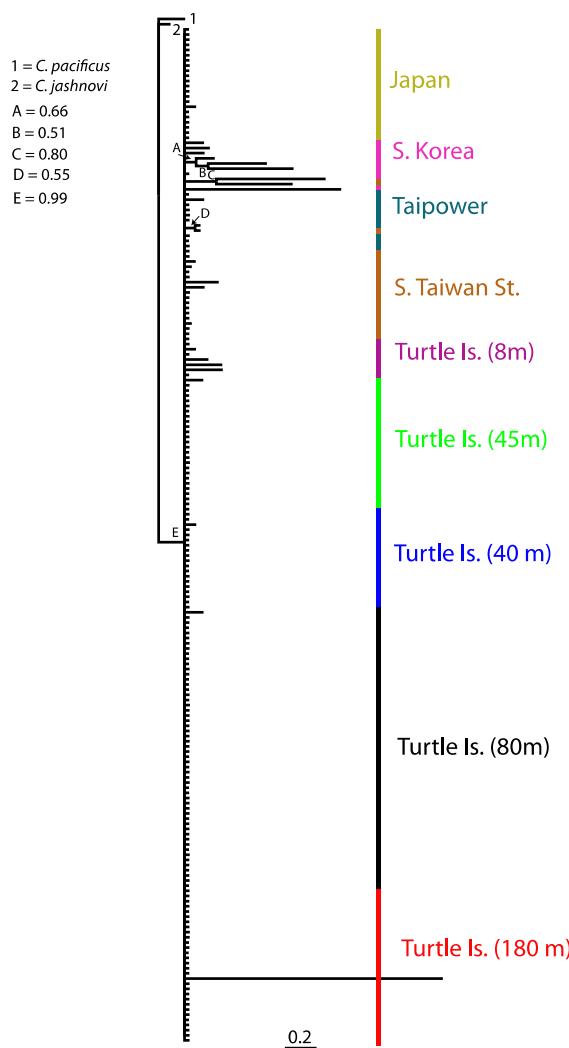


Fig. 4 Bayesian phylogenetic tree based on ITS-1 sequences of *Calanus sinicus*. Samples from different locations have been color-coded for clarity

sinicus from Japan were pooled from different depth zones (0–50 and 200–400 m), quite possibly masking any differences related to depth. Population genetic analysis of ITS-1 indicates that the deeper samples (180 m) from Turtle Island, Taiwan, are genetically more related to samples away from Turtle Island than those from the more shallow depths from Turtle Is. (Table 4). If these genetically distinct samples represent reservoirs of deeper populations of *C. sinicus* during summer, then it is possible that depth contributes to the population structure of *C. sinicus*. However, no depth partitioning of the genetic population structure was observed with the cytb gene. Cytb data

indicate that there is sufficient random mixing among *C. sinicus* inhabiting surficial waters down to 180 m depth. The significance of depth in shaping genetic differentiation has been corroborated by Goetze (2011) who estimated the genetic structure of the diel vertical migration (DVM) of the calanoid copepod *Pleuromamma xiphias* throughout its global distribution, and compared these results to those expected, given the interaction of this species' habitat depth with ocean circulation and bathymetry. We are limited to properly test the hypothesis of genetically distinct reservoir population in deeper waters in *C. sinicus* by the absence of more locations with depth stratified samples and larger sampling effort.

The discordant patterns of population differentiation between the two genes are puzzling. Generally, more variation is expected at the mitochondrial genes of most metazoans, however, in most sampling stations, ITS-1 was more variable than cytb (Table 1). Our understanding of this observation would have improved with higher number of sequenced samples in some locations (e.g., in S. Korea, Turtle Is. 8 m). Some of these discrepancies may also be explained by the atypical sequence evolution of ITS-1. ITS-1 may be a useful ribosomal region for sequence comparisons within and between closely related species when concerted evolution has homogenized the variability among the ribosomal repeats within individuals and species (Hillis and Dixon, 1991). However, if the concerted evolution is partial or slow, multiple divergent copies of ITS-1 may exist within individuals and species (Vogler & Desalle, 1994; Harris & Crandall, 2000) and inferences based on ITS-1 sequences may be misleading (Harris & Crandall, 2000; Marquez et al., 2003).

Calanus sinicus is a species that originates from the ECS (Hwang et al., 2006) and the winter assemblage of this copepod is demarcated by high Indicator Value Index numbers (Chen, 1992). Otherwise, these are temperate copepods that are commonly found in waters around Taiwan (Shih & Young, 1995; Hwang et al., 2004; Hwang et al., 2006; Dur et al., 2007; Tseng et al., 2008d). Other oceanographic (e.g. currents) and biological factors (e.g. diapause) may influence the genetic population structure of *Calanus sinicus* and these have not been taken into account in the present study. Coastal but also deep water currents throughout the known distribution of *C. sinicus* should be taken into consideration since *C. sinicus* can be found as

deep as 1,000 m (Nonomura et al. 2008). Diapause has been reported in *C. sinicus* from Japan (Nonomura et al. 2008). However, how widespread and at what times this metabolic activity is present in most populations of *C. sinicus* is unknown. More extensive sampling that includes Bohai Sea as well as samples from its southernmost distribution (e.g., waters of Hong Kong and Hainan Island) will improve our understanding of the genetic population structure of this important member of mesozooplankton.

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