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Genetic variability and divergence of *Neutrodiaptomus tumidus* Kiefer 1937 (Copepoda: Calonida) among 10 subpopulations in the high mountain range of Taiwan and their phylogeographical relationships indicated by mtDNA COI gene

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Abstract

Background: In the mountain area of Taiwan, we investigated 10 subpopulations of *Neutrodiaptomus tumidus* Kiefer 1937 living in isolated alpine ponds or lakes. We used mitochondrial DNA cytochrome C oxidase subunit I (COI) sequence as molecular marker to investigate the population genetic structure and their phylogeographical relationships.

Results: We obtained 179 sequences from 10 subpopulations and found 94 haplotypes. Nucleotide composition was AT-rich. Haplotype diversity (Hd) and nucleotide diversity (π) indicated significant genetic differences between subpopulations (Hd = 0.131 ~ 0.990; π = 0.0002 ~ 0.0084); genetic differentiation index (F_{st}) and gene flow index (N_m) also exhibited significant genetic diversification between subpopulations (F_{st} = 0.334 ~ 0.997; N_m = 0 ~ 1). Using Tajima's D and Fu and Li's $D*$ and $F*$ as neutrality tests, we found that the nucleotide variation within the population was consistent with the neutral theory except in the JiaLuoHu subpopulation. The JiaLuoHu subpopulation significantly deviated from the neutral theory and was speculated to have experienced a bottleneck effect. According to the phylogenetic tree, these alpine lake subpopulations could be divided into four phylogroups (northern region, Xueshan group, central region, and southwestern region). Xueshan group contains one subpopulation, DuRongTan, which is a unique group relative to other groups. It is close to northern group geographically but closer to southwestern group genetically. According to AMOVA, the major genetic variation came from different geographical distribution of subpopulations. Molecular clock estimates that the northern and southern regional divergence time was about 2.2 ~ 3.9 MYA, when the Central Mountain Range uplift (3 ~ 5 MYA) caused the population of *N. tumidus* to be segregated into northern and southern parts.

Conclusions: Significant genetic divergence between each subpopulation of *N. tumidus* was found in this study. This result indicated the low dispersion ability of planktonic copepods with limited gene flow between each subpopulation.

Keywords: Metapopulation; Population genetics; Phylogeography; Copepoda; mtDNA; COI

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Background

Based on the geological record, Taiwan, an island separated from mainland China by the Taiwan Strait, emerged above sea level not more than 500 million years ago (MYA). About 300 MYA, the first uplifted land appeared as the earliest stage of the Central Mountain Range (Teng 2002). After the formation of the Central Mountain Range, other series of mountain formations formed Xueshan Range, Yushan Range, and Alishan Range (Figure 1). Today these four major mountain systems cover the two third of the land area of Taiwan; the Central Mountain Range stretches along the central area of island from north to south and off to the east. Along the west side of central mountain ridge are the Xueshan Range and Yushan Range. West of Alishan and slightly further south is the Yusan Range. The northern part of Xueshan Range is more isolated from the main range, and the LanYang River system flows through the deep valley between Xueshan Range and Central Mountain Range and northward into Ilan Bay. DaJia River system runs across middle region of Xueshan Range then through the western plain into the Taiwan Strait, with its headwaters near the origin of LanYang River. Zhuoshui
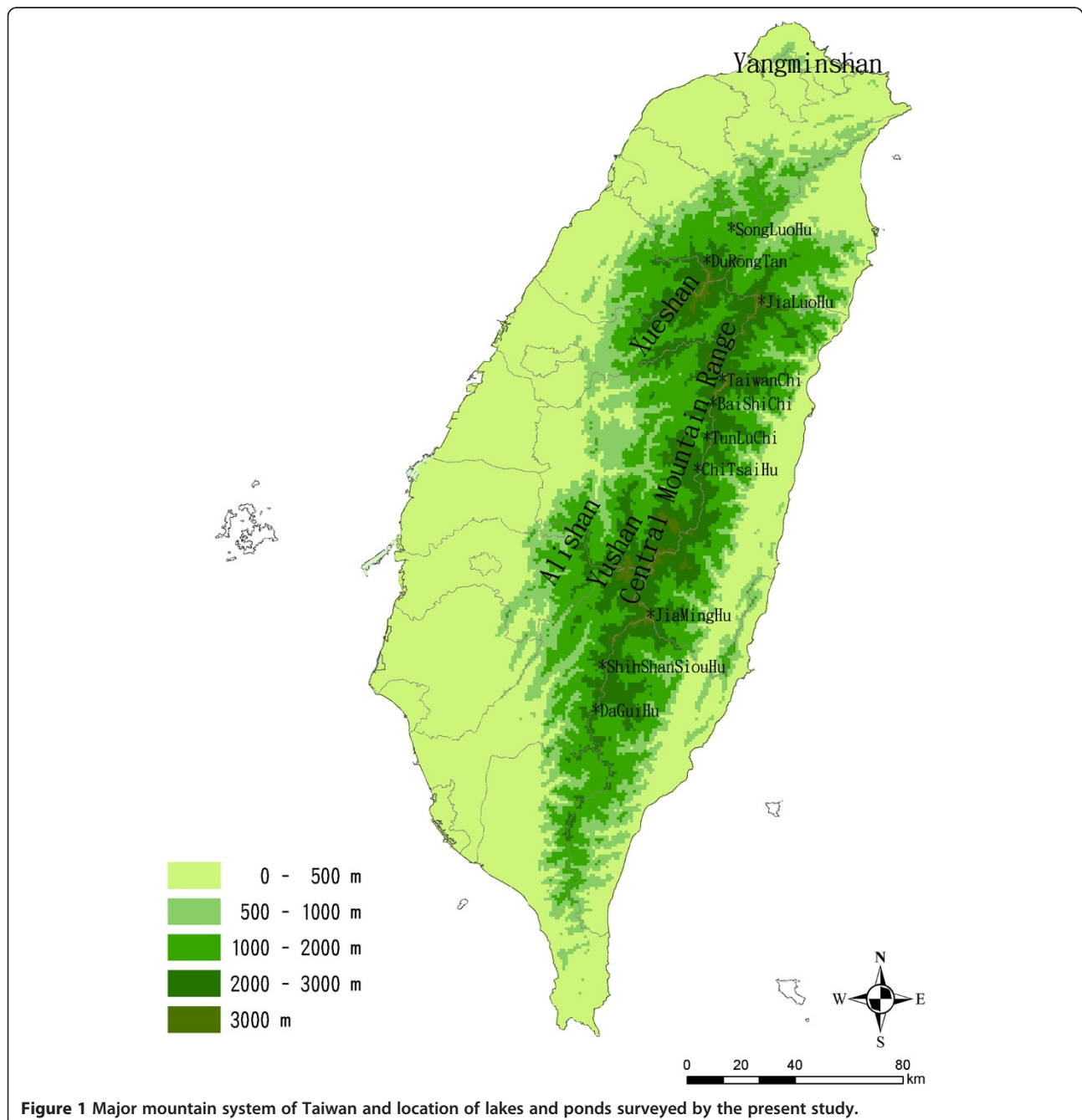
Figure 1 Major mountain system of Taiwan and location of lakes and ponds surveyed by the present study.

River, originated from the Central Mountain Range (HeHuanShan), also flows westward and through the mountains between Xueshan and Yushan Ranges into the Taiwan Strait.

Besides rivers, there are small lakes and ponds scattered in remote areas of the mountains. According to Chen and Wang (1997), the high-mountain water body of Taiwan can be divided into two types: type I is above the forest line on the alpine grassland and type II is at the lower altitudes with dense forest around the water body. Lakes or ponds above the forest line usually are small in size and isolated from the river system without clear connection, their volume of water is much fluctuated seasonally, and some of them are dried out during long period without rain. Lakes and ponds in the forest are normally larger in size and deeper than those of the type I and always have clear connection to the river system; their volume of water also fluctuated seasonally but never dried out. Disregarding the types, lakes and ponds are important natural habitats for all kinds of wildlife as well as human beings.

Some general ecological survey of highland lakes and ponds of Taiwan had been conducted in the past (Kano and Yoshimuva 1934; Otsu et al. 1988, 1989; Otsu et al. 1992a, b; Lin et al. 2006, 2011a, b). Otsu et al. (1988) reported the presence of *Eudiaptomus formosus* from DaGuiHu (Big Ghost Lake) (Taromalin in Japanese) in southern Taiwan. However, Young and Shih (2011) surveyed Taromalin in 1997 and 2008 and only found *Neutrodiaptomus tumidus*. Besides Taromalin, Young and Shih (2011) also found *N. tumidus* in freshwater bodies within the mountain area, with altitudes ranging from 1,300 to 3,500 m and remote from human activity. All the taxonomic surveys on the mountain area also found many species of Cladocera, cyclopoid, and harpacticoid copepods (Young 2010; Young and Tuo 2011; Tuo and Young 2011). Some populations of *N. tumidus* inhabit in small ponds and lakes higher than the forest line and have absolutely no water connection among each other. Some populations live in type II water bodies and are isolated by different river systems. To investigate the isolation and dispersal of these scattered populations of *N. tumidus*, studies on their metapopulation genetics can be useful.

In the metapopulation model, each subpopulation is considered as a small pool in a large area, and the population dynamics is based on colonization-extinction equilibrium between each subpopulation (Hanski 1998, 1999). Freshwater invertebrates, including copepods, living in lakes or ponds not always connected to each other by water way in high mountain area are strong candidates for metapopulations. According to Bohonak and Jenkins (2003), if there is a metapopulation in freshwater invertebrate populations, dispersal studies play a crucial role in quantifying the effects of individual migration. Dispersal of individuals can inhibit extinction of subpopulation and maintain genetic diversity. The genetic divergence studies between each subpopulation can provide basic evidence of dispersion and estimation of the gene flow rate.

In this study, we report the levels of genetic variability and divergence within and among 10 subpopulations of *N. tumidus* isolated in lakes and ponds in the high mountain area. We screened sequence of mtDNA coding for a fragment of cytochrome C oxidase subunit I (COI). This gene has been proven informative at the interspecific and intraspecific levels in crustacean (Henzler 2006; Kelly et al. 2006; Ketmaier et al. 2010; Pavesi et al. 2011; Young et al. 2012). More specifically, first we tested whether the geographical isolation of lakes and ponds will prevent gene flow or not, and second we constructed the phylogeographical relationships of the subpopulations.

Methods

Ten samples were taken using a plankton net with mesh size of 55 μm. All the collection sites were isolated from each other by long distance and absence of water connection (Figure 1). Samples were fixed in 70% ethanol (EtOH) immediately after collection then transferred to 95% EtOH and stored at a low temperature (less than –20°C). Within 72 h, each sample was sorted and identified under a stereomicroscope. Based on sampling sites, 10 populations were collected (Table 1). Individuals in each population were randomly selected for the analysis of their genetic structure by COI gene marker.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from single animals using Chelex (InstaGene Matrix BIO-RAD 7326030, Bio-Rad Laboratories, Hercules, CA, USA). Each specimen from 95% EtOH was taken and placed in pure water for 1 h for cleaning. Each of the cleaned specimen was placed at the bottom of a 0.5-ml centrifuge tube for 30 min to dry in a speed vacuum-drying system. Dried specimens were then crushed by needles and incubated in 30 μl of 5% Chelex solution at 56°C for 2 to 3 h to extract the DNA then continued for 8 min at 90°C. For each polymerase chain reaction (PCR), 5 μl of upper cleaning solution was used as the DNA template after centrifugation at 10^4 rpm ($9,168 \times g$) for 3 min.

We employed the universal primers, LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2918 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994), to amplify the mitochondrial COI gene by a PCR. For the population of DaGuiHu, HCO2198 and LCO1490 could not be successfully applied. We designed another primer DGF (5′-TGTTGGTATAGAAT GGGATCTC-3′) and DGR (5′-GACTTTGTATTTAA TCGCCGG-3′) based on the sequence amplified from HCO2198 and LCO1490. Each PCR sample had a total

Table 1 Sampling location, sample size, and collection date of 10 populations of *Neutrodiaptomus tumidus*

Geographic distribution (mountain range)	Population site (code)	Altitude (m)	Sample size (N)	Collection date
Xueshan	SongLuoHu (SL)	1,300	15	February 2009
Xueshan	DuRongTan (DR)	2,350	18	May 2009
Central mountain	JiaLuoHu (JL)	2,320	30	April 2005
Central mountain	TaiwanChi (TC)	2,900	15	November 2009
Central mountain	BaiShiChi (BS)	2,770	15	November 2009
Central mountain	TunLuChi (TL)	2,850	15	November 2009
Central mountain	ChiTsaiHu (CT)	2,900	15	February 2010
Central mountain	JiaMingHu (JM)	3,310	18	September 2011
Central mountain	ShihShanSiouHu (SS)	2,300	25	April 2011
Central mountain	DaGuiHu (DG)	2,150	13	January 2008
Total			179	

The locality names are phonetically translated by the common use romanization system.

volume of 50 μl and consisted of pH 9.2 buffer solution (50 mM Tris-HCl, 16 mM ammonium sulfate, 2.5 mM $MgCl_2$, and 0.1% Tween 20), 5 pM of each primer, 50 μM of dNTPs, 2 units of *Taq* DNA polymerase (super Therm DNA polymerase, *Bio-Taq*, BioKit Biotechnology, Miaoli, Taiwan), and 10 to 50 ng of genomic DNA. The PCRs were performed in an Eppendorf Mastercycler gradient 384 machine (Eppendorf, Hamburg, Germany). Thermocycling began with 5 min of preheating and continued with 35 cycles at 94°C for 30 s, primer annealing at 51°C for 45 s, and extension at 72°C for 45 s, followed by incubation at 72°C for 10 min for full extension of the DNA and ended with 4°C holding. PCR products were electrophoresed in 2% agarose gels, after which the gels were stained with ethidium bromide (EtBr) and photographed under an ultraviolet light box. DNA fragments were excised from the gel and extracted using a 1-4-3 DNA extraction kit (Gene-Spin, Protech, Taipei, Taiwan) to obtain purified DNA. Sequences of DNA fragments were resolved on an ABI3730 automated sequencer (Applied Biosystems, Carlsbad, CA, USA) using 20 to 50 ng of template with 5 pM of the LCO1490 and HCO2198 primers read from both directions.

Alignment, genetic diversity, and phylogeny

COI gene sequences were aligned by eye using the SeqMan (DNASTAR, Lasergene version 7.1, 2006). We calculated the haplotype diversity (Hd, Nei 1987), nucleotide diversity (π, Nei 1987), genetic differentiation indices (F_{st}, Hudson et al. 1992), gene flow index (N_m, Hudson et al. 1992), genetic distance (D_{xy}, Nei 1987; average genetic distances between each pair of species), and neutrality test (including Tajima *D* test (Tajima 1989), Fu and Li's *D** and Fu and Li's *F** (Fu and Li 1993)), using DNAsp version 5.10 (Rozas 2009). The index of neutrality test which equals to 0 indicated that

the population genetic structure reaches to neutral expectation. The positive value indicates the population genetic structure under some type of balancing selection or diversifying selection. The negative value indicates that an excess of rare alleles is selected against some genotypes or population under growing condition with excess rare alleles (Hartl and Clark 2007). The COI sequences of *Mongolodiaptomus birulai* (GenBank accession number AB592995) and *Neodiaptomus schmackeri* (GenBank accession number AB592987) from Lin (2004) and Young et al. (2013) were used as out groups, and the phylogeographical tree was derived using all sequences by the maximum likelihood (ML), maximum-parsimony (MP), and Bayesian inference (BI). The maximum likelihood analysis was run by MEGA version 6 (Tamura et al. 2013) using model GTR + G with 100 runs and found the best ML tree by comparing the likelihood scores. The robustness of the ML tree was evaluated by 1,000 bootstraps. Maximum-parsimony methods (Saitou and Nei 1987) were based on Kimura two-parameter (K2P) distances with 1,000 bootstraps using MEGA version 6 (Tamura et al. 2013).

We also used MrBayes version 3.1.2 (Huelsenbeck et al. 2001) to run BI. Before BI analyses, the model was selected by MrModeltest 3.7 (Posada and Crandall 2001), and the optimal evolutionary parameters was selected by Akaike information criterion (AIC) from MrModeltest 3.7. The obtained best models were GTR + I + G and HKY + I + G, respectively. The search was run with four chains for 10 million generations and four independent runs, with trees sampled every 1,000 generations. The convergence of the chains was determined by the effective sample size (ESS) (>200 as recommended) in Tracer (v. 1.5, Rambaut and Drummond 2009), and the first 1,000 trees were discarded as the burnin (determined by the average standard deviation

of split frequency values below the recommended 0.01; Ronquist et al. 2005).

The minimum spanning network among each population was constructed by TCS version 1.21 (Clement et al. 2000). Between populations and geographic groups, the analyses of molecular variance (AMOVA) were executed by Arlequin version 3.1 (Excoffier and Schneider 2005) to verify the variance between each subpopulations in the same geographic groups and different geographic groups. For each subpopulation, the diversification level was estimated by the index of Harpending (1994) and Ramos-Onsins and Rozas (2002). Harpending's raggedness index was estimated to test whether the sequence data from each population deviated from what is expected under a sudden expansion, which is based on mismatch distributions of pairwise differences among all haplotypes in a sample. Ramos-Onsins and Rozas's $R2$ neutrality tests by comparison of the difference between the number of singleton mutation and the average number of nucleotide differences were conducted.

Results
Analysis of genetic diversity
We had 179 sequences (individuals) in total from 10 subpopulations: 13 sequences from DaGuiHu (DG) with length of 608 bp and the other 166 sequences from nine other subpopulations with length of 658 bp. The mean nucleotide composition was adenine 23.7% (22.9% to 24.5%), thymine 35.7% (34.5% to 37.0%), cytosine 17.3% (16.3% to 18.5%), and guanine 23.3% (22.3% to 24.0%). The AT-rich COI segment contained 59.4% of nucleotides as A + T. Based on 608 bp comparison, the average ratio of transition/transversion was 3.38 and not approximated to saturation. The 608 bp of COI segments were composed of 453 sites without variation and 155 polymorphic sites. Out of 155 polymorphic sites, 123 were parsimony informative sites and the other 32 were singleton

variable sites. The variable sites included 21 on the first codon (15.4%); the second codon is without variable site, and variable sites on third codon are 115 (86.6%).

Among the 10 subpopulations, 179 individuals belonged to 94 haplotypes (accession number AB921860-AB921953); the Hd was 0.966 ± 0.00006 (range 0.990 to 0.131) and π was 0.0782 (range 0.084 to 0.0002) (Table 2). Each subpopulation had unique haplotype that was not shared with other subpopulations. The haplotype diversity of BaiShiChi (BS) (13 haplotypes) and ChiTsaiHu (CT) (14 halpotypes) are 0.990 ± 0.028 higher than other subpopulations, and their nucleotide diversity was 0.0072 and 0.0084, respectively. The haplotype diversity of JiaLuoHu (JL) (three halpotypes) and TaiwanChi (TC) (two halpotypes) populations was much lower than other subpopulations, with haplotype diversity of 0.131 and 0.133 and nucleotide diversity of 0.0003 and 0.0002, respectively.

Population genetic structure of *N. tumidus*
Among the 10 subpopulations, ranges of genetic differentiation indices (Fst) between each subpopulation pair were from 0.334 to 0.997 (with 3 subpopulation pairs less than 0.9 and mean of 0.948), which indicated high differentiation between the subpopulations. According to Fst, the mean number of genes (N_m) exchanged between each subpopulation pair was 0.03 (range 0 to 1), and between the closest subpopulation, BS and TC only had one copy of gene exchanged from one generation. Based on Kimura's two-parameter model estimation, the range of intersubpopulation genetic distance between each pair was from 3.3 ± 0.7% to 14.8 ± 1.8%, and the intrasubpopulation genetic distance between each individuals was less than 1% (Table 3). Across different species and genera, the genetic distance, e.g., 27% to 30% between *N. tumidus* and outgroup *Neodiaptomus schmackeri* and 23% to 36% between *N. tumidus* and outgroup *Mongolodiaptomus birulai*, was much larger than interpopulation differences.

Table 2 Sample size, haplotype number, Hd, π, and polymorphic sites of populations of *N. tumidus*

Site (sample size)	No. of haplotype (accession number)	Haplotype diversity (Hd) ± SD	Nucleotide diversity (π)	Polymorphic site (S)	Singletons
SL (15)	10 (AB921916-AB921925)	0.95 ± 0.040	0.0042	11	5
DR (18)	5 (AB921908-AB921912)	0.60 ± 0.113	0.0013	5	4
JL (30)	3 (AB921913-AB921915)	0.31 ± 0.082	0.0003	3	3
TC (15)	2 (AB921952-AB921953)	0.13 ± 0.112	0.0002	1	1
BS (15)	13 (AB921860-AB921872)	0.99 ± 0.028	0.0072	21	14
TL (15)	11 (AB921941-AB921951)	0.95 ± 0.040	0.0071	17	8
CT (15)	14 (AB921887-AB921900)	0.99 ± 0.028	0.0084	29	19
JM (18)	14 (AB921873-AB921886)	0.94 ± 0.003	0.0045	21	22
SS (25)	15 (AB921926-AB921940)	0.95 ± 0.001	0.0042	18	14
DG (13)	7 (AB921901-AB921907)	0.81 ± 0.113	0.0068	19	11
Total (179)	94 (AB921860-AB921953)	0.97 ± 0.000	0.0078	155	32

Table 3 Pairwise comparisons of genetic distance (below diagonal and diagonal) and standard error (above diagonal) between populations of *N. tumidus*

	SL (%)	DR (%)	JL (%)	TC (%)	BS (%)	TL (%)	CT (%)	JM (%)	SS (%)	DG (%)
SL	0.4	1.7	0.7	1.6	1.6	1.6	1.7	1.5	1.8	1.7
DR	11.4	0.1	1.6	1.2	1.5	1.5	1.6	1.4	1.5	1.5
JL	3.3	11.8	0.0	1.6	1.5	1.5	1.6	1.5	1.7	1.8
TC	11.7	7.1	11.8	0.0	0.8	0.8	0.9	0.8	1.7	1.8
BS	12.6	9.8	11.4	4.4	0.7	0.3	0.5	0.9	1.5	1.7
TL	12.1	9.8	11.1	4.0	1.1	0.7	0.5	0.9	1.5	1.8
CT	13.3	11.1	12.4	5.2	2.0	2.0	0.9	1.0	1.6	1.8
JM	11.6	8.8	12.0	4.3	5.7	5.6	6.7	0.5	1.6	1.7
SS	14.7	9.5	14.1	11.4	11.3	11.5	12.6	11.3	0.4	1.1
DG	14.5	11.0	14.3	13.3	13.4	14.0	14.8	14.1	7.5	0.6

The genetic distance was calculated using Kimura two-parameter model.

The genetic distance (GD) between each subpopulation pair was correlated significantly with geographical distance (ID) (GD = 0.000395 ID + 0.062, $R = 0.62$, $F = 26.4$; $p < 0.000$). Geographical distance of more than 100 km separation can reach more than 10% genetic difference. Based on the result of neutrality test (Table 4), the genetic variation of whole populations was under the random effect of mutation or genetic drift (Tajima's $D = 1.05$, $p > 0.1$; Fu and Li's $D* = 0.32$, $p > 0.1$; Fu and Li's $F* = 0.80$, $p > 0.1$). For each subpopulation, three of them with genetic differentiation deviate from neutral state; thus, the population might have expanded recently and was under bottleneck effect or founder effect. These three subpopulations were JL (Tajima's $D = -1.73$, $0.05 < p < 0.1$; Fu and Li's $D* = -2.69$, $p < 0.05$; Fu and Li's $F* = -2.79$, $p < 0.05$), JiaMingHu (JM) (Tajima's $D = -2.34$ $p < 0.01$; Fu and Li's $D* = -2.37$, $p < 0.01$; Fu and Li's $F* = -3.56$, $p < 0.01$), and ShihShanSiouHu (SS) (Tajima's $D = -1.39$, $05 < p < 0.1$; Fu and Li's $D* = -2.66$, $p < 0.05$; Fu and Li's $F* = -2.80$, $p < 0.05$).

Table 4 Neutrality tests of each population of *N. tumidus*

| Population	Tajima's *D*	Fu and Li's *D**	Fu and Li's *F**
SL	−0.98	−0.76	−0.94
DR	−1.27	−1.92	−2.00
JL	−1.73	−2.69*	−2.80*
TC	−1.16	−1.43	−1.54
BS	−1.10	−1.60	−1.69
TL	−0.62	−0.80	−0.86
CT	−1.78	−1.71	−2.00
JM	−2.34**	−3.37**	−3.56**
SS	−1.80	−2.66 *	−2.80*
DG	−1.39	−1.02	−1.27
Total	1.05	0.32	0.80

*$p < 0.05$; **$p < 0.01$.

Analysis of phylogeographical relationships

Phylogeographical relationships between each subpopulation pair can be divided into four phylogeographical groups based on ML analysis, MP analysis, and BI. Three analysis methods reach similar dendrograms (Figure 2). Statistic support (ML/MP/BI) on each node was represented by bootstrap values with 1,000 replicates. Outgroups were *Mongolodiaptomus birulai* (MB) and *Neodiaptomus schmackeri* (NS). The northern phylogeographical group included SongLuoHu (SL) and JL (supported value, ML 100%; MP 99%; BI 97%), in both lakes located in northern Taiwan but of different mountain system. DuRongTan (DR) is a unique population among all subpopulations and is located near the northern group geographically, but its genetic component was closer to the southwestern group (supported value ML 72%; MP 77%; BI 92%). The central phylogeographical group included TC, BS, TunLuChi (TL), CT, and JM (supported value, ML 99%; MP 81%; BI 95%). These geographically related series of small water bodies are located in the Central Mountain Range in the middle of Taiwan. Among these phylogeographical groups, JM is closer to southwestern group geographically. The southwestern phylogeographical group included SS and DG (supported value, ML 99%; MP 95%; BI 97%), in both lakes at the southern end of Central Mountain Range.

The major difference of the relationship between phylogeographical groups derived by ML, MP, and BI is the linkage of the northern group. The northern group of ML and MP tree was an independent clade (supported value 100% and 99%), and BI analysis result with northern group as a base of tree could not divide it into an independent clade.

The minimum spanning network analysis showed no significant result for all the subpopulations. Of all subpopulations, only the geographically closely related ones, BS, TL, and CT, can share a significant minimum spanning network with BS as the expanding center. These
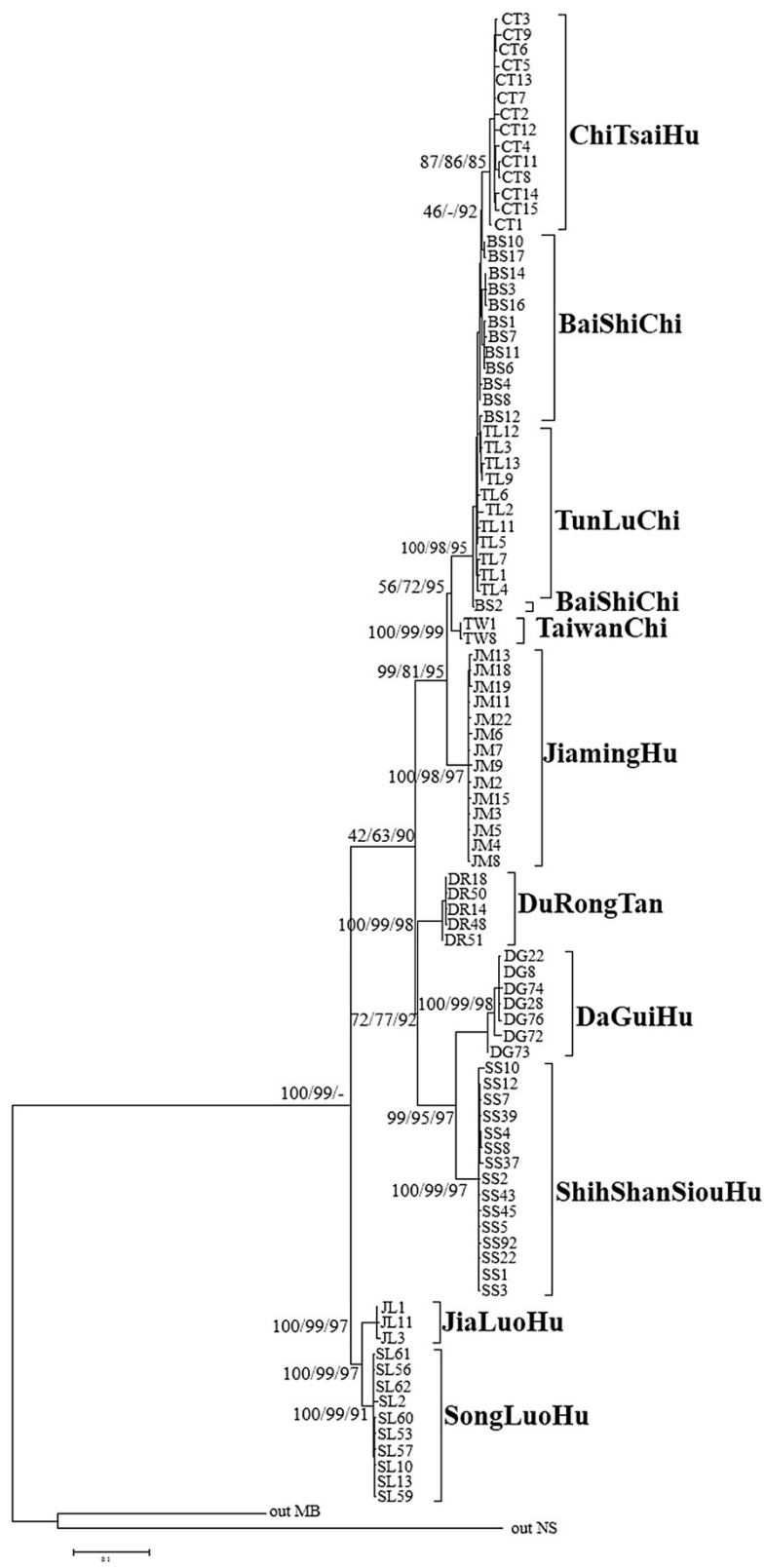
Figure 2 Maximum likelihood tree based on COI partial sequence variation among all haplotypes of *N. tumidus*.

three subpopulations, geographically from north to south and with less than 10 km apart between two neighboring subpopulations, are all in the field of the Yushan bamboo, *Yushania niitakayamensis*, of the Central Mountain Range.

Analysis of molecular variance and molecular clock

According to the four phylogroups derived by ML, MP, and BI methods, the analysis of molecular variance between each pair of subpopulation indicated that 60.35% of the genetic variation belonged to intergroup difference, 36.07% variation to the population within group, and 3.58% variation within the populations (Table 5). The genetic differentiation was significant among phylogroups ($\Phi = 0.603$, $p < 0.0001$), between each subpopulation within a phylogroup ($\Phi = 0.964$, $p < 0.0001$), and within a population ($\Phi = 0.909$, $p < 0.0001$).

By analysis of pairwise differences among all DNA sequences in a population, the mismatch distribution indicated that JL, TW, and DR subpopulations are still in the state of range expansion (Harpending's raggedness index = 0.65, 0.56, 0.12) (Table 6). The other subpopulations are in stationary state (Harpending's raggedness index less than 0.08), and the mismatch distribution as single peak indicated ancient separation and expansion of population. The mismatch distribution of $R2$ statistics that was closer to 0 indicated that the population process matched with the predicted expansion model with big population size.

We use the gene diversification rate of marine *Sesarma* (1.66% per million year) and terrestrial *Sesarma* (2.33% per million year) as a baseline to estimate the molecular clock of *N. tumidus* evolution. The diversification between DG-DR and SL-JL was the oldest, at 2.8 to 3.9 MYA, DG and DR at 2.2 to 3.0 MYA, and separation of SL-JL and central group at about the same time of first diversification between DG and SL.

Discussion

The moisture from Pacific Ocean and South China Sea intercepted by mountain plays a crucial role of water supply for lakes or ponds on forest edge or higher than timberline. Even in long-time dry season without rain, the shrank water body has still water enough for aquatic organisms year after year. *N. tumidus* lives in highland

Table 5 Analysis of molecular variance (AMOVA) of *N. tumidus* among different populations

Source of variation	df	Percentage of variation	Φ value	p value
Among groups	3	60.35	0.60346	<0.0001
Among populations within groups	6	36.07	0.96417	<0.0001
Within populations	169	3.58	0.90964	<0.0001

df, degrees of freedom.

Table 6 Analysis of pairwise differences among all DNA sequences in a population and mismatch distribution for each population

	Harpending's raggedness index (r)	Ramos-Onsins and Rozas (R_2) statistic
SL	0.074	0.091
DR	0.123	0.107
JL	0.651	0.132
TC	0.556	0.249
BS	0.039	0.085
TL	0.036	0.110
CT	0.044	0.068
JM	0.041	0.047
SS	0.056	0.067
DG	0.074	0.093

waters with water temperature reaching 4°C during the 4- to 5-month cold period and not higher than 15°C throughout the year. Based on our laboratory observation, its individuals still can actively swim when the water temperature was subzero prior to ice formation. During our transportation of samples from collecting sites in the mountain to laboratory, they can survive in wet mud with limited water for many days under low temperature. Most lakes and ponds of our sampling sites are acidic, with pH value less than 6 (Chen and Wang 1997), mainly due to decomposition of organic matter and acid rain. The most acidic waters were in SL, JL, and CT. DG has the largest water volume and a pH value close to 7. The aquatic organisms that live in high elevation and shallow water face another challenge, the UV radiation. The red color in *N. tumidus* was from carotenoid pigments deposited in oil drop and protects the animal from UV damages (Hairston 1980). A relatively harsh abiotic environment, the highland water bodies are devoid of fish and other larger predators and, therefore, provide an environment with relatively low predation pressure. Consequently *N. tumidus* is able to maintain a high population density in these waters throughout the year. During our survey, our plankton net was frequently clogged by the reddish copepods in any season.

Mitochondria DNA evolved in rapid way; the gene order of copepods is very different from other invertebrates (Machida et al. 2002; Rawson and Burton 2006). Some studies also present high variation of COI sequence in interpopulation level in different geographical scales (Lee 1999; Rawson and Burton 2006; Chen and Hare 2008). These properties of mitochondria gene may explain why Folmer's primers fail to work on DG subpopulation.

Based on the water volume filtered by plankton net, we could find 10 or even more individuals of *N. tumidus* per liter of water and estimated that each subpopulation

size to be over millions and even billions. Grant and Bowen (1998) suggested that if Hd > 0.5 and π > 0.005, the population was stable with a huge population size. The whole population of *N. tumidus* in Taiwan with Hd = 0.966 > 0.5 and π = 0.0078 > 0.005 fits with this category. The subpopulations such as BS, TL, CT, and DG also have Hd > 0.5 and π > 0.005. The subpopulations SL, DR, JM, and SS with Hd > 0.5 and π < 0.005 might, according to Avise (1994) and Grant and Bowen (1998), experience bottleneck effect first, then accumulated genetic variation and increased haplotype diversity but has limited mutation to increase nucleotide diversity through expansion of population. The subpopulations JL and TW with Hd < 0.5 and π < 0.005 might experience long-term bottleneck effect and founder effect to expedite evolution without accumulating too much variation even with large population size.

Among our sampling sites, only two were connected to river, DG as one of the headwaters of LaoNong River at southern Taiwan and SL as one of the headwaters of Nan-Shih-Si River, upstreams of Tanshui River system at northern Taiwan. Except DG and SL, other sampling sites were fully isolated on the mountain ridge. The river system may be a two-way traffic for some aquatic animals, such as fishes and aquatic insects, but a one-way traffic (down the stream) for zooplanktons. Geographical isolation, limited dispersal ability, and low gene flow between subpopulations have been the major cause of the independent evolution among subpopulations. The most geographical distant group between DG-SS and SL with genetic distance greater than 14% is subjected to difference at species level as suggested by many authors for different crustacean groups (Adamowicz and Purvis 2005; Adamowicz et al. 2007; Costa et al. 2007; Markow and Pfeiler 2010).

According to Bofkin and Goldman (2007), the evolution rate of the second codon was much lower than the third codon for most families. Base on the codon redundancy, the second-codon position is the most functionally constrained; any change to the second-codon position causes a nonsynonymous change in the coding sequence. The third-codon position is the least functionally constrained. This may explain why the COI coding sequence of *N. tumidus* has no second-codon variation. In the COI coding sequence, *N. tumidus* has 86.6% variable sites on the third codon, and the translated AA sequences only have less than 1.2% differences between each pair of subpopulations, similar to their limited difference between external morphology (Young and Shih 2011). The diversification of external morphology less than their COI sequence may reflect their harsh living environment with similar abiotic factors such as low temperature, acidity of water, and high UV radiation. The genetic differences based on COI sequences could be

an appropriate evidence for long-term isolation between each population without gene flow. If we justify the species identification only by sequences differences without morphological comparisons, some subpopulations will treat as a subspecies or a new species.

JL subpopulation with genetic differentiation deviates from neutral state based on neutrality test (Tajima's *D*, Fu and Li's *D** and Fu and Li's *F**) and still in the state of range expansion as indicated by Harpending's raggedness index and Ramos-Onsins and Rozas statistic. According to the satellite image, JL was a group (11 pools) of water bodies in different sizes which may or may not connect each other. We could only sample two of them, and some pools are even untouched by human until now. The population ecology of these sites still needs more study to understand its relationship between pools. This site also maybe the best place to study the metapopulation process of zooplankton without frequent human disturbances.

The large genetic divergence found among subpopulations of *N. tumidus* suggests that these subpopulations have begun their diversification about 2.2 to 3.9 MYA, matching the geological formation of mountain system in Taiwan about 500 to 300 MYA (Teng 1990; Liu et al. 2000). Based on the geological formation of mountain system in Taiwan, the landmass was first lifted in the north then extended southward (Suppe 1981), and the expanding population of *N. tumidus* should follow the same pattern of geological formation. The phylogeographic relationships in this study also represent the history of metapopulation evolution. The phylogenetic tree morphology indicates that the northern and central phylogeographic group shared a common ancestor and their sister group, DR, shared a common ancestor with the southern phylogeographic group. DR population might be the descendants of an ancient population which was genetically close to DG and SS. We speculate that diversification of the *N. tumidus* population began at the formation of Xueshan Range. One lineage of this ancient population extended southward along the Central mountain ridge and another lineage northward along Xueshan Range. Prior to the end of the last glacial period, extinction of subpopulations occurred in high elevation site, and Xueshan Range and the south end of the central mountain became a *refugium* for *N. tumidus*. After the glacial epoch, the present subpopulations at Central Mountain Range, excluding DG and SS, originated from north end of Xueshan. Subpopulations DG and SS were not disperse northward, and all the central area was inhabited by populations dispersed southward from the northern *refugium*. Some studies of plants with limited mobility also consider Xueshan Range and south end of central mountain as a *refugium* during the Ice Age (Hwang et al. 2003: *Cunninghami konishii*;

Huang et al. 2004: *Trochodendron aralioides*; Cheng et al. 2006: *Castanopsis carlesii*).

The taxonomic status of *N. tumidus* and their possible relationships with *Neutrodiaptomus formosus* had been discussed by Young and Shih (2011). The close-related *N. formosus* is an endemic species of Japan that lives in lakes with low water temperature. Some other *Neutrodiaptomus* could be found in more northern part and as a dominant species (Kurenkov 1970: *Neutrodiaptomus angustilobus*, Kamchatka). *Neutrodiaptomus* in China also not a common species, it could found only in some isolated lake on remote area (Shen et al. 1979). The distribution scenario of *Neutrodiaptomus* is more or less like the distribution of *N. tumidus* subpopulation in Taiwan. Our study only can unravel part of history of *N. tumidus* just in Taiwan; the origin of this species in Taiwan may have more ancient phylogeographical story connected to other *Neutrodiaptomus* species living in Japan and Eurasia continent.

Conclusions

In this study, we found significant genetic divergence between each subpopulation of *N. tumidus* isolated in high mountain ponds or lakes. This result indicated the low dispersion ability of planktonic copepods with limited gene flow between each subpopulation for a long time. The formation of these in the water body and maintaining a high population density for a long period of time was a great mystery and needed to be studied more. The phylogeographical relationship between each subpopulation indicated by COI gene sequences presents multiple divisions along chronological scale. The oldest subpopulation is located at northern Taiwan, which shared the close relationship with the southern subpopulation by early division. Both old lineages were all located at lower elevation considered to be the *refugium* during a glacial period: the high-latitude northern population at about 1,500 m and the low-latitude southern population up to 2,000 m. Subpopulation colonization from north to south on higher elevation at about 3,000 m happened more recently with complicated division pattern. More geological studies for lakes and ponds on high mountain range about their formation times are necessary to match the pattern of phylogeographical relationship of *N. tumidus*. Some other invertebrates live in the same sites with different dispersion ability could also be candidates for study in the future to result more solid facts about this page.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
SSY designed the experiment, collecting samples, data analysis and drafted the manuscript. YYL and MYL carried out the molecular genetic studies, participated in the sequence alignment and phylogeographical analysis. All authors read and approved the final manuscript.

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