Freshwater Biology

Freshwater Biology (2013) doi:10.1111/fwb.12152

Evolutionary history of alpine and subalpine *Daphnia* in western North America

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SUMMARY

1. Studying adaptation within a multispecies complex can be challenging due to uncertainty about the evolutionary relationships among populations. An accurate phylogenetic context is especially important for drawing conclusions about phenotypic evolution for species used as model organisms in ecological and evolutionary research.

2. Within the zooplankton genus *Daphnia*, carapace melanin pigmentation has often been suggested as an adaptation to ultraviolet radiation exposure in clear-water habitats. However, the evolutionary history of melanism has been obscured because of phylogenetic uncertainty.

3. We inferred a molecular phylogeny based on the mitochondrial ND5 and COI loci and used it to explore the evolutionary history of populations belonging to the *Daphnia pulex* species complex that inhabit alpine and subalpine lakes and ponds in western North America.

4. Our analysis confirms the existence of a distinct mitochondrial clade for *Daphnia melanica* within the *D. pulex* species complex and shows it to be more geographically widespread than previously thought and includes populations from several disjunct sites.

5. We show that melanism, once used as a diagnostic character for identifying *Daphnia* species, is polymorphic within the *D. melanica* clade. This polymorphism suggests the possibility of independent parallel evolutionary losses and gains of melanism in this species complex.

Keywords: *Daphnia melanica*, *Daphnia pulex*, melanism, molecular phylogeny, UV radiation

Introduction

Studying adaptation and evolutionary divergence among natural populations of organisms requires knowledge of the historical relationships among groups. These relationships exist across scales, ranging from genetic structure among closely related subpopulations of a single species to phylogenetic relationships among species, genera or higher taxonomic levels. To accurately describe and measure phenotypic evolution requires a known phylogenetic context (Felsenstein, 1985). By its nature, studying organismal evolution involves explicit and implicit comparisons among populations of the same species and among close relatives. Minimally, the species identity of focal populations and a well-supported phylogeny for those species is a prerequisite. Even for within-species investigations that do not incorporate the comparative method, knowing the taxonomic identity of the focal species is critical to generating hypotheses, designing experiments and making comparisons with existing literature.

Establishing phylogenetic context, and even identifying species, can be difficult for certain classes of organisms, especially those that hybridise readily and frequently. This difficulty is typical of many zooplankton such as cladocerans and rotifers that exhibit both sexual and asexual reproductive modes. These taxa reproduce by cyclic parthenogenesis, in which clonal reproduction by females is typical during optimal conditions, and males are produced and sexual reproduction occurs in

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response to ecological cues such as waning day length and/or diminished food supply (Lampert, 2011). Frequent hybridisation among species during bouts of sexual reproduction can make species delimitations difficult, even when employing multiple methods. Morphological characters have often proven unreliable (Benzie, 2005) despite their widespread use in the recent past. While the introduction of molecular phylogenies led to a revolution both in species concepts and in our approach to phylogenetic questions (Huelsenbeck *et al*., 2001; Felsenstein, 2004), molecular phylogenies can often be misleading or in conflict. For frequently hybridising taxa, reticulate evolution and incomplete lineage sorting can lead to stark disagreement between mitochondrial and nuclear phylogenies, and among nuclear phylogenies based on different loci (Vergilino *et al*., 2011; Cristescu *et al*., 2012).

The *Daphnia pulex* species complex has been extensively studied for several decades because of its predominance in North American fresh waters (Benzie, 2005) and the tractability of *Daphnia* as an ecological and molecular model system (Miner *et al*., 2012). The genetic structure of this complex has been difficult to resolve definitively due to lack of diagnostic morphological characters and the tendency of species to hybridise. However, portions of the mitochondrial *NADH dehydrogenase* subunit 5 (ND5) and *cytochrome c oxidase* subunit I (COI) loci have often been used for elucidating relationships among groups (Colbourne *et al*., 1998; Weider *et al*., 1999; Weider & Hobæk, 2003; Markova *et al*., 2007; Mergeay *et al*., 2008; Adamowicz *et al*., 2009; Vergilino *et al*., 2011; Crease *et al*., 2012; Cristescu *et al*., 2012). In fact, the mitochondrial ND5 phylogeny is currently the most widely used tool for evaluating species identity for new samples that belong to the *D. pulex* complex. For example, it has been used recently to demonstrate both the Nearctic origin of a subset of European *Daphnia pulicaria* lineages (Markova *et al*., 2007), a possible South American origin of the entire North American *D. pulicaria* clade (Mergeay *et al*., 2008), and to show that panarctic *D. pulex* has a nearly worldwide distribution (Crease *et al*., 2012). These molecular phylogenetic insights are a cautionary tale for researchers studying adaptation and evolutionary process in *Daphnia*, because morphologically uniform 'species' have repeatedly been shown to possess cryptic evolutionary subdivisions. Were *Daphnia* not a long-standing model species with growing popularity among genome biologists (Colbourne *et al*., 2011; Miner *et al*., 2012), unappreciated diversity within populations would perhaps be of trivial interest. The widespread use of these species, especially those in the *D. pulex* complex, to test hypotheses about ecological and evolutionary process dictates that researchers evaluate the phylogenetic context of each population studied.

Pigmented populations in the *D. pulex* complex that occupy clear-water habitats with pronounced exposure to ultraviolet radiation (UVR) have a particularly interesting and vexing history and have variously been assigned to *D. pulex*, *D. pulicaria*, *D. middendorffiana*, *D. tenebrosa* and *D. melanica*. From the perspective of evolutionary process, carapace pigmentation (hereafter, 'melanism') is a fascinating phenotype because it occurs in geographically widespread and disjunct habitats, from temperate coastal sand dunes to high arctic localities around the world. These habitats share the common environmental stressor of UVR exposure due to minimal concentrations of light-absorbing dissolved organic matter in the water. Carapace pigmentation is composed of melanin (Hebert & Emery, 1990) and has photoprotective properties that provide a survival advantage under UVR exposure (Hebert & Emery, 1990; Hessen, 1996). In addition, melanism has been assumed to be costly because the phenotype is plastic: animals lose pigmentation when no longer exposed to UVR (Tollrian & Heibl, 2004; Hansson, Hylander & Sommaruga, 2007; Scoville & Pfrender, 2010), and melanic forms can have lower growth rates than non-melanic forms (Hessen, 1996). Despite the existence of a host of interesting ecological and evolutionary studies concerning the occurrence, induction and genetic control of pigmentation in the *D. pulex* complex (e.g. Hessen, Borgeraas & Orbaek, 2002; Hansson *et al*., 2007; Scoville & Pfrender, 2010), the evolutionary history of the phenotype is uncertain. Is melanism an ancestral or derived phenotype? Has it evolved multiple times independently? Has it ever been lost in a lineage, or does plasticity preserve the trait from being lost in populations that do not need it? We can only explore these questions within an appropriate phylogenetic context.

Carapace pigmentation is widespread in Arctic *Daphnia* and was historically used to delimit species, with pigmented forms assigned to *Daphnia middendorffiana* and unpigmented form*s* assigned to *D. pulex* (Brooks, 1957). However, it has been known for several decades that the taxonomic situation is more complex for Nearctic *Daphnia*. Pigmented forms have been documented in populations molecularly identified as *D. pulex*, primarily in arctic North American populations. A general correlation has also been documented between the increased occurrence of both polyploidy and pigmentation at higher latitudes (Beaton & Hebert, 1988; Van Raay & Crease, 1995). *D. middendorffiana* is pigmented in most studies, and it is now known to be a polyploid obligate

asexual lineage (Hebert & McWalter, 1983; Hebert & Emery, 1990; Dufresne & Hebert, 1994). Furthermore, pigmented lineages of *D. pulicaria* (identified with ND5 sequence) have been documented in the European and North American arctic (Markova *et al*., 2007), in addition to other melanic European lineages that have been variously identified as *D. pulex*, *D. tenebrosa* or *D. middendorffiana based* on morphology alone (Hessen, 1996; Hessen *et al*., 1999, 2002; Hansson *et al*., 2007). To further complicate the discrimination of melanic forms, the narrow endemic pigmented species *Daphnia melanica* was introduced (but not formally described) based on allozyme electrophoresis of individuals from a single locality in coastal Oregon (Hebert, 1995). More recently, the *D. melanica* species designation has been applied to *Daphnia* in both the Sierra Nevada (previously identified as *D. middendorffiana* based on morphology) and Olympic Mountains of western North America without presentation of supporting data for this taxonomic designation (Fisk *et al*., 2007; Latta *et al*., 2007, 2010; Knapp & Sarnelle, 2008; Scoville & Pfrender, 2010; Miner & Kerr, 2011).

Here, we present molecular phylogenies derived from mitochondrial sequence data for populations within the *D. pulex* complex from clear-water habitats in both arctic and temperate alpine localities in North America. We test the hypothesis that *D. melanica* forms a distinct and well-supported clade, within which melanism is polymorphic (Scoville & Pfrender, 2010; Miner & Kerr, 2011). We seek to confirm the validity of the designation of *D. melanica* as an independent lineage based on allozymes (Hebert, 1995) and to illuminate the complex evolutionary history of UVR-tolerant lineages. Future studies concerning adaptation in the *D. pulex* complex should always be addressed within a phylogenetic context as we do here, particularly when melanism is involved.

Methods

Sampling locations

We collected *Daphnia* belonging to the *D. pulex* morphotype from lakes and ponds in North America, including localities in arctic Canada, Beartooth Mountains of Montana, U.S.A., Olympic Mountains of Washington, U.S.A., and Sierra Nevada of California, U.S.A. (Table 1). We sampled water bodies from an inflatable raft with a handheld plankton tow net either via horizontal (shallow ponds) or vertical (deeper lakes) tows. *Daphnia* were preserved on-site in 95% ethanol and stored at −20°C on return to the laboratory. The populations sampled had previously been morphologically identified as *D. middendorffiana* (Sierra Nevada; Stoddard, 1987; Knapp, Matthews & Sarnelle, 2001), or *D. pulex*, or were labelled as *D. melanica* based on unpublished sequence data (Fisk *et al*., 2007; Latta *et al*., 2007; Scoville & Pfrender, 2010; Miner & Kerr, 2011). Sierra Nevada populations had previously been shown to be melanic in fishless lakes (Scoville & Pfrender, 2010), whereas all Olympic populations are non-melanic (Miner & Kerr, 2011).

DNA sequencing

We extracted DNA from individual field-collected *Daphnia* to ensure that each DNA sample represented a single clone rather than a mixture of multiple genotypes. We used the CTAB DNA extraction method (Cristescu *et al*., 2006) and the polymerase chain reaction (PCR) to amplify portions of the mtDNA COI and ND5 genes using primers COIf (5′ -TCT CAA CTA CTC ATA AGG ACA TTG G-3′), COIr (5′ -TAT ACT TGA GGA TGA CCA AAG AAC CA-3′), ND5a (5′ –AAA GAA GAA ACC ATA TTA AAC C-3′) and ND5b (5′-GGG GTG TAT CTA TTA ATT CG-3′). Amplifications were performed in 50 μL reactions using 2 μL of genomic DNA extraction, 5 μL PCR buffer with MgCl2, 5 μL 8 mM dNTP mix, 5 μL of each 2 μM primer, 0.2 μL Taq DNA polymerase (PerkinElmer, Waltham, MA, USA) and water to final volume. Reactions consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 92°C for 45 s, 46°C for 60 s and 72°C for 90 s and a final extension step at 72°C for 5 min. Negative controls were performed with all reactions. PCR products were purified using Qiaquick PCR purification spin columns (QIAGEN, Valencia, CA, USA). The DNA sequences of purified PCR products were generated using Dye Terminator Cycle Sequencing (Life Technologies, Grand Island, NY, USA) and run on an ABI 3730 automated DNA sequencer (Life Technologies). PCR products were sequenced from both the forward and reverse directions to generate overlapping fragments, which were assembled into contiguous sequences and aligned using SEQUENCHER 5.0 (Gene Codes, Ann Arbor, MI, USA). All sequences were deposited in GenBank under accession numbers KC831965-KC831998 (ND5) and KC831999-KC832033 (COI). We also included in our analyses previously existing DNA sequence data from earlier publications (Colbourne *et al*., 1998; Crease, 1999; Paland & Lynch, 2006; Mergeay *et al*., 2008; Vergilino, Belzile & Dufresne, 2009; Latta *et al*., 2010; Vergilino *et al*., 2011). The final alignment of 1089 bp comprised 591 bp of COI and 498 bp of ND5 for 58

Table 1 Sample information for all sequences used in the analyses. Species ID abbreviations are as follows: EuroPUL: European *Daphnia pulicaria*, EuroPX: European *D. pulex*, Mel: *D. melanica*, Midd: *D. middendorffiana*, NearPUL: Nearctic *D. pulicaria* and PanPX: Panarctic *D. pulex*. Citation abbreviations are as follows: Colb98: (Colbourne *et al*., 1998), Crease99: (Crease, 1999), Latta10: (Latta *et al*., 2010), Merg08: (Mergeay *et al*., 2008), Pal06: (Paland & Lynch, 2006), Verg09: (Vergilino *et al*., 2009), Verg11: (Vergilino *et al*., 2011), JKC: specimens provided directly by John K. Colbourne. Genbank accessions denoted with 'X' represent sequences that were not available, while 'none' means that the sequences are not archived in Genbank but were obtained directly from the author of the citation

				Genbank Accession Nos.	
Code	Locality	Species ID	Reference	ND5	CO1
Dpu_ EU152310	Germany	EuroPUL	Merg08	EU152310	EU152330
Dpu_ EU152307	Czech Republic	EuroPUL	Merg08	EU152307	EU152322
Dpx_ EU152308	Czech Republic	EuroPX	Merg08	EU152308	EU152321
Dpx_ EU152309	Belgium	EuroPX	Merg08	EU152309	EU152320
Beartooth_P8	Beartooth Mtns., U.S.A.	Mel	This study	KC831968	KC832002
Dmel_DQ340844	Oregon, U.S.A.	Mel	Pal06	X	DQ340844
Dmel_ORE 1	Oregon, U.S.A.	Mel	Colb98	None	X
Dmel_ORE 2	Oregon, U.S.A.	Mel	Colb98	None	X
Dmel_ORE 3	Oregon, U.S.A.	Mel	Colb98	None	X
Dmel_ORE 4	Oregon, U.S.A.	Mel	Colb98	None	X
Olympic_C3	Olympic Mtns., U.S.A.	Mel	This study	KC831979	KC832013
Olympic_C4	Olympic Mtns., U.S.A.	Mel	This study	KC831987	KC832021
Olympic_E3	Olympic Mtns., U.S.A.	Mel	This study	KC831984	KC832018
Olympic_E4	Olympic Mtns., U.S.A.	Mel	This study	KC831985	KC832019
Olympic_M3	Olympic Mtns., U.S.A.	Mel	This study	KC831986	KC832020
Olympic_M4	Olympic Mtns., U.S.A.	Mel	This study	KC831983	KC832017
Olympic_R4	Olympic Mtns., U.S.A.	Mel	This study	KC831988	KC832022
Olympic_Y3	Olympic Mtns., U.S.A.	Mel	This study	KC831978	KC832012
Olympic_Y4	Olympic Mtns., U.S.A.	Mel	This study	KC831980	KC832014
Olympic_Z3	Olympic Mtns., U.S.A.	Mel	This study	KC831981	KC832015
Olympic_Z4	Olympic Mtns., U.S.A.	Mel	This study	KC831982	KC832016
S_ALC006	Sierra Nevada, U.S.A.	Mel	This study	KC831976	KC832010
S_EVE001	Sierra Nevada, U.S.A.	Mel	This study	KC831972	KC832006
S_GOL001	Sierra Nevada, U.S.A.	Mel	This study	KC831973	KC832007
S_LOG006	Sierra Nevada, U.S.A.	Mel	This study	KC831971	KC832005
S_MAR001	Sierra Nevada, U.S.A.	Mel	Latta10	HM137667	HM131366
S_MSK002	Sierra Nevada, U.S.A.	Mel	Latta10	HM137677	HM131376
S_PUP001	Sierra Nevada, U.S.A.	Mel	This study	KC831975	KC832009
S_SNO001	Sierra Nevada, U.S.A.	Mel	Latta10	HM137687	HM131386
S_SOU001	Sierra Nevada, U.S.A.	Mel	Latta10	HM137697	HM131396
S_SQU002	Sierra Nevada, U.S.A.	Mel	Latta10	HM131406	HM137707
S_UPS001	Sierra Nevada, U.S.A.	Mel	This study	KC831974	KC832008
S_WAH001	Sierra Nevada, U.S.A.	Mel	Latta10	HM137717	HM131416
Beechy_8	Beechy Island, Canada	Midd	This study	KC831966	KC832000
Bracebridge_1	Bracebridge Inlet, Canada	Midd	This study	KC831996	KC832031
Creswell_17	Creswell Bay, Somerset Is., Canada	Midd	This study	KC831994	KC832029
Creswell_5	Creswell Bay, Somerset Is., Canada	Midd	This study	KC831965	KC831999
Dmid_DOF 14	District of Franklin, Canada	Midd	Colb98 + JKC	None	None
Freeman_31	Northwest Territories, Canada	Midd	This study	KC831991	KC832026
Longstaff_314	Longstaff Bluff, Baffin Is., Canada	Midd	This study	KC831989	KC832023
Longstaff_39	Longstaff Bluff, Baffin Is., Canada	Midd	This study	KC831967	KC832001
Rolwey_9	Rowley Is., Canada	Midd	This study	KC831992	KC832027
Aston_10	Aston Bay, Somerset Is., Canada	NearPUL	This study	KC831998	KC832033
Aston_11	Aston Bay, Somerset Is., Canada	NearPUL	This study	KC831970	KC832004
Beartooth_P9	Beartooth Mtns, U.S.A.	NearPUL	This study	KC831997	KC832032
Beechy_4	Beechy Island, Canada	NearPUL	This study	KC831995	KC832030
Creswell_1	Creswell Bay, Somerset Is., Canada	NearPUL	This study	KC831977	KC832011
Dpu_FJ591103	Indiana, U.S.A.	NearPUL	Verg09	FJ591103	X
Dpu_FJ591104	Indiana, U.S.A.	NearPUL	Verg09	FJ591104	X
Dpu_FJ591112	Québec, Canada	NearPUL	Verg09	FJ591112	X
Dpu_FJ591124	Québec, Canada	NearPUL	Verg09	FJ591124	X

Table 1 (Continued)

Code	Locality	Species ID	Reference	Genbank Accession Nos.	
				ND5	CO1
Dpu_HQ434690	Winnipeg, Manitoba, Canada	NearPUL	Verg11	HQ434690	X
FlintLake_1	Flint Lake, Baffin Is., Canada	NearPUL	This study	X	KC832024
Iglootik_94	Igloolik Is., Canada	NearPUL	This study	KC831969	KC832003
Ricksbury_30	Northwest Territories, Canada	NearPUL	This study	KC831990	KC832025
Ricksbury_33	Northwest Territories, Canada	NearPUL	This study	KC831993	KC832028
Dpx_DQ340836	Michigan, U.S.A.	PanPX	Pal06	DQ340836	DQ340836
Dpx_NC000844	Ontario, Canada	PanPX	Crease99	NC000844	NC000844

samples (Table 1). We estimated the number of segregating sites and the number of synonymous and non-synonymous substitutions in our alignments using DnaSP v5 (Librado & Rozas, 2009).

Phylogenetic inference

We employed jModelTest version 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) to select the simplest adequate model of nucleotide substitution for each locus independently, basing our selection on values of Akaike's information criterion corrected for small sample size (AICc). We evaluated 24 different nucleotide substitution models for each locus, based upon the three substitution schemes JC, HKY and GTR, and including options for unequal base frequencies (+F), a proportion of invariable sites (+I), gamma-distributed rate variation among sites (+G), and all combinations of these options. We then inferred phylogenies both via Bayesian inference using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) and by maximum likelihood with bootstrapping using RAxML 7.2.8 (Stamatakis, 2006; Stamatakis, Hoover & Rougemont, 2008) on the XSEDE platform provided by the CIPRES Science Gateway (Miller, Pfeiffer & Schwartz, 2010). For both methods of phylogenetic inference, we partitioned the data set to separate the two loci and allowed for different models of nucleotide substitution within each partition. In both programs, we specified a European *D. pulicaria* sequence from Mergeay *et al.* (2008) (Dpu_EU152310, first row of Table 1) as the outgroup.

For our partitioned Bayesian analysis in MrBayes, we used the best-fit substitution models determined by jModelTest (GTR+G for COI and HKY+G for ND5) and allowed all parameters, including the overall substitution rate, to differ between partitions. We included four independent runs with random starting trees and ran for 2×10^6 generations, which resulted in a final average standard deviation of split frequencies of 0.009036. We sampled trees every 1000th generation and discarded the first 25% of trees as burn-in. We then generated a majority-rule consensus tree and calculated the posterior probability at each node as the proportion of trees that included the node.

In our partitioned maximum-likelihood (ML) analysis using RAxML, we used GTR+G substitution models ('GTRGAMMA') for both partitions but allowed parameters to differ between partitions. Although jModelTest identified the HKY+G substitution model as the best fit for the ND5 data, because the HKY model is itself a simplification of the GTR model, it is reasonable to apply the GTR model to these data. We conducted rapid bootstrapping using the GTRCAT approximation with 25 distinct rate categories, and estimated confidence levels on the final ML tree using 1000 bootstrapped replicates.

Results

We collected sequence data for a 498-bp fragment of the mitochondrial ND5 locus and a 591-bp fragment of the mitochondrial COI locus from 35 individual field-collected *Daphnia* (Table 1). For phylogenetic context, we included existing sequence data from 23 additional *Daphnia* samples in our analysis, including representatives of each of the taxonomic groups identified by earlier researchers (European *D. pulicaria* and *D. pulex*, Nearctic *D. pulicaria*, Panarctic *D. pulex* and *D. middendorffiana*; Table 1; Colbourne *et al.*, 1998; Crease, 1999; Paland & Lynch, 2006; Mergeay *et al.*, 2008; Vergilino *et al.*, 2009, 2011). For some of these additional samples, both ND5 and COI sequences were available; for others, only ND5 sequence data exist (Table 1). Also among the existing sequences were Sierra Nevada samples published previously (Latta *et al.*, 2010). Both gene regions, COI and ND5, were aligned to open reading frames. No premature stop codons or indels were noted for either region. The alignment of the COI region contained 138 segregating sites with 134 synonymous and 22 non-synonymous substitutions. The alignment of the ND5

gene region contained 117 segregating sites with 103 synonymous and 21 non-synonymous substitutions.

We inferred majority-rule consensus phylogenetic trees via Bayesian inference (Figs 1 & 2) and ML with bootstrap replication (Fig. 3) in analyses that partitioned the two loci and allowed differing rate parameters using the nucleotide substitution models described above. All of our samples fell within the well-supported non-European *D. pulex/pulicaria* clade (Fig. 1), so we focus on this region of the phylogeny from now on (Figs 2 & 3). We find that all Olympic and Sierra Nevada samples belong to the *D. melanica* clade (Figs 2 & 3, grey shading), and the arctic samples belong to either the Neartic *D. pulicaria* clade or the *D. middendorffiana* clade (Figs 2 & 3). The two samples from the Beartooth Mountains of Montana are a unique case: one of these belongs to the *D. melanica* clade ('Beartooth P9'), while the other falls within the Neartic *D. pulicaria* ('Beartooth P8') in both Bayesian and ML trees (Figs 2 & 3).

Although Olympic and Sierra Nevada samples all belong to the well-supported *D. melanica* clade and represent sister taxa in this analysis, existing data show them to be divergent in the melanism phenotype. Sierra Nevada populations from fishless lakes are highly melanised (Scoville & Pfrender, 2010), but all Olympic populations have low melanin content, well under half that of the Sierra Nevada populations (Miner & Kerr, 2011). Olympic populations are of course still capable of synthesising melanin (as in all *Daphnia* species, melanin is present in the eye and ephippium), but it is not deposited in the carapace nearly to the same degree as in Sierra Nevada animals (Miner & Kerr, 2011).

Discussion

Our analysis confirms the existence of the mitochondrial *D. melanica* clade within the *D. pulex* species complex. The *D. melanica* subdivision was first identified

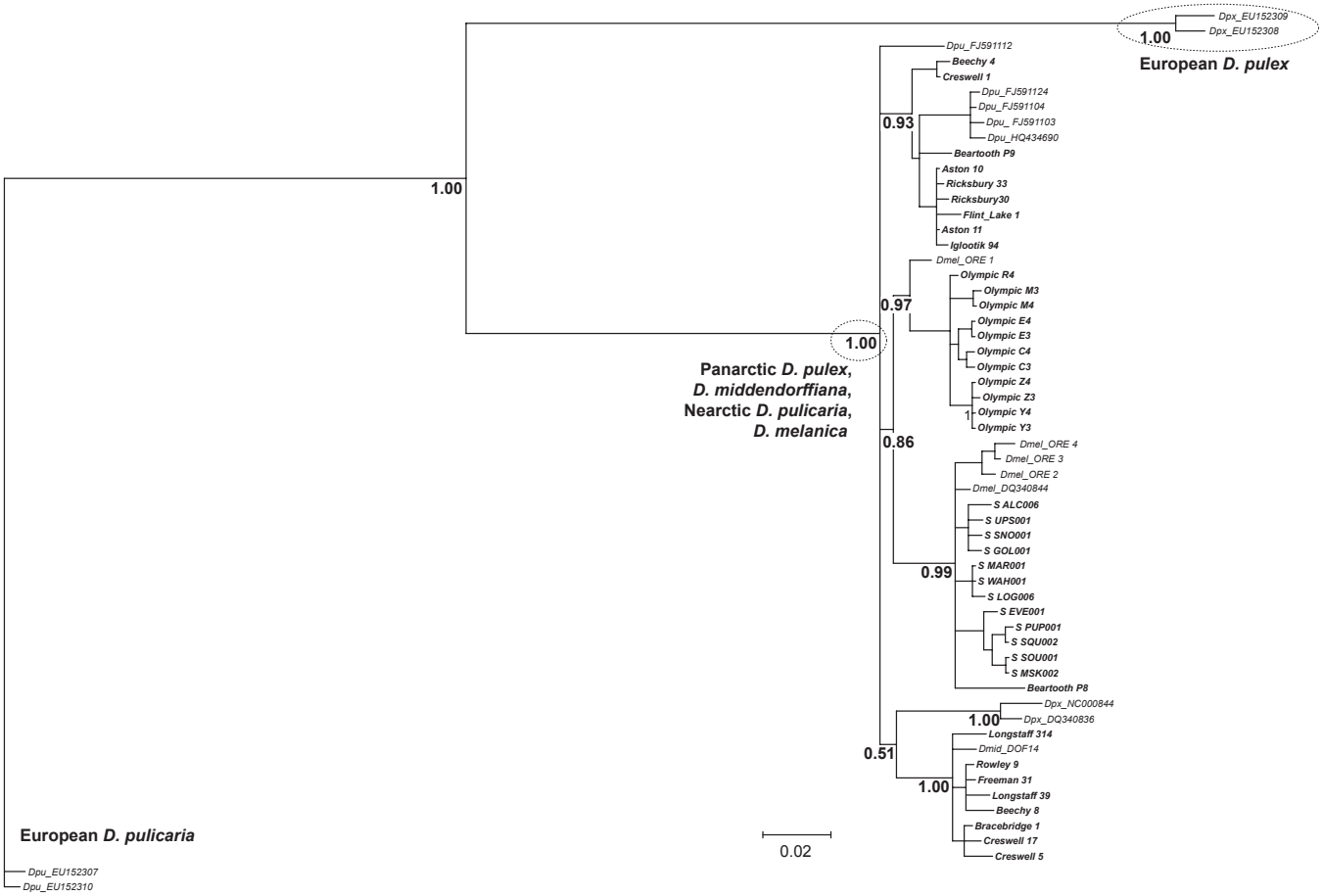

Fig. 1 Phylogeny of the complete data set generated under Bayesian inference. Bold sample names indicate those samples newly collected and analysed for this study. Numbers at selected nodes denote posterior probabilities. There is much more phylogenetic distance among European lineages of *Daphnia pulicaria* and *D. pulex* than there is among the North American lineages present in the large polytomy with short branch lengths.

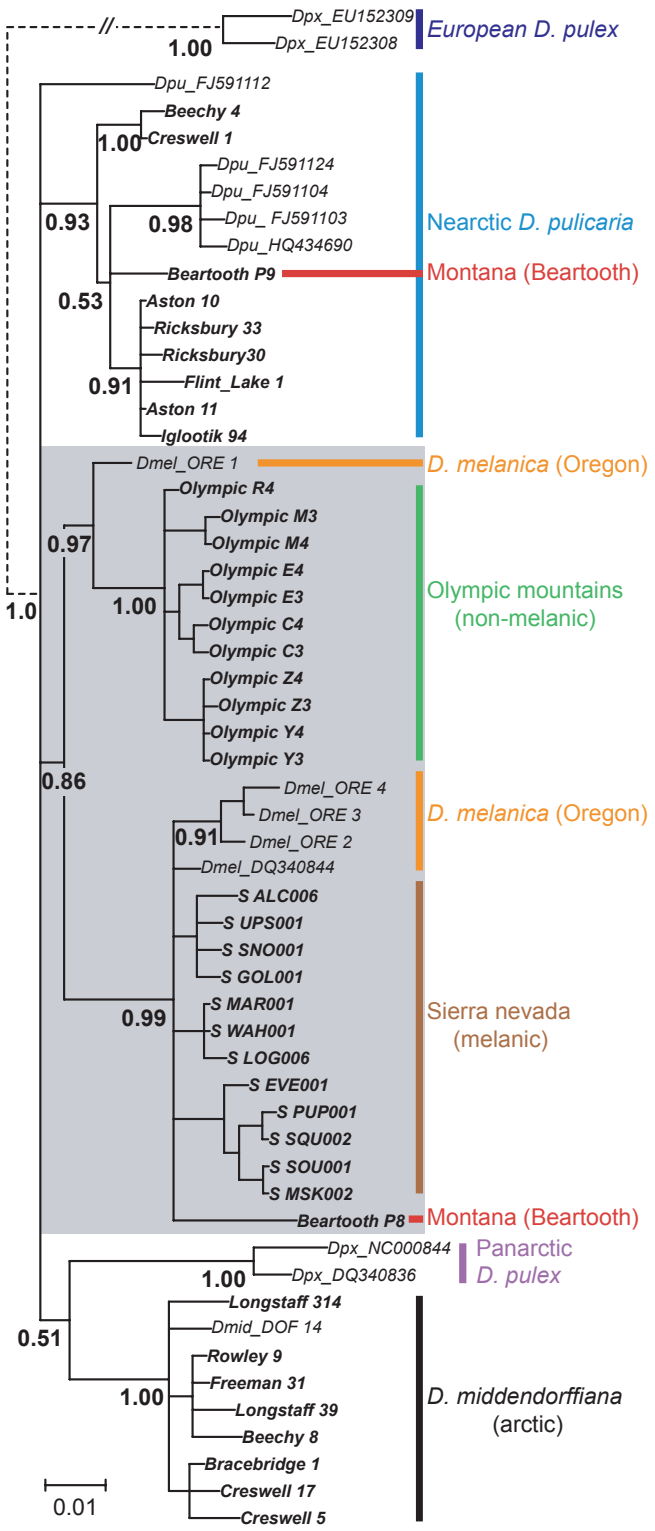

Fig. 2 The portion of the complete Bayesian phylogeny from Fig. 1 that contains all North American lineages. The *Daphnia melanica* clade is shaded in grey. Numbers at select nodes represent posterior probabilities as in Fig. 1. Sampling localities are denoted by coloured bars and labels at right. Bold sample names indicate those samples newly collected and analysed for this study.

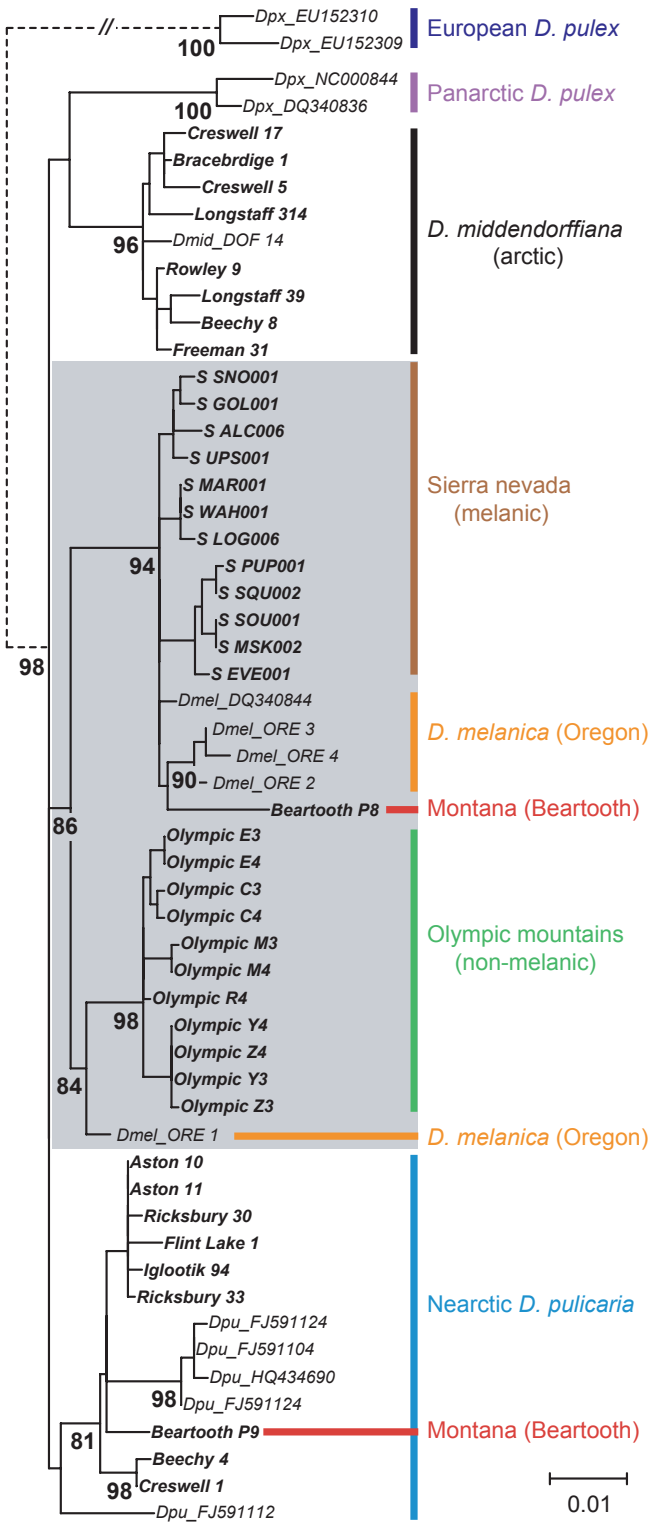

Fig. 3 North American portion of the best-scoring maximum-likelihood (ML) phylogeny generated by RAxML, analogous to Fig. 2 but generated by ML rather than Bayesian inference. Numbers at select nodes represent the percentage of bootstrap replicates (out of 1000) that contained the node; bootstrap support values below 80 are not shown. Grey shading identifies the *Daphnia melanica* clade.

and named over 15 years ago (Hebert, 1995) and was supported by both mitochondrial sequence and allozyme data (Crease *et al*., 1997; Paland, Colbourne & Lynch, 2005). However, until recently, the only populations of *Daphnia* thought to belong to this group were located in a geographically restricted area in western Oregon (Crease *et al*., 1997; Colbourne *et al*., 1998). Recent studies have identified the Sierra Nevada and Olympic Mountain populations as belonging to the *D. melanica* clade without providing supporting data (Fisk *et al*., 2007; Latta *et al*., 2007, 2010; Knapp & Sarnelle, 2008; Scoville & Pfrender, 2010; Miner & Kerr, 2011). Here, we include these populations in a detailed phylogenetic analysis based on mitochondrial sequence data.

Although phylogenetic inference within the *D. pulex* complex can be complicated by a history of reticulate evolution leading to conflict between mitochondrial and nuclear loci genealogies (Vergilino *et al*., 2011; Cristescu *et al*., 2012), we provide a testable phylogenetic framework and acknowledge that additional information derived from genomewide patterns of variation may ultimately alter our perspective. In our analysis, *D. melanica* forms a well-supported monophyletic group that includes the original 'type' populations from western Oregon along with subalpine populations in the Olympic Mountains and alpine populations in the Sierra Nevada and Beartooth Mountains. This species does not extend further north than the Beartooth Mountains in our samples, since there were no *D. melanica* present in our North American Arctic samples. However, a definitive understanding of the range of this species is limited by our sampling scheme. Since most known populations are in alpine or subalpine habitats, further examination of high-elevation regions across North America is clearly warranted. One notable feature of the *D. melanica* tree is that all but one of the Oregon genotypes are more closely related to the Sierra Nevada populations than to populations in the Olympic Mountains. That two specimens from the same pond (ORE-1 and ORE-2 from Colbourne *et al*., 1998) fall into different clades, one with Sierra Nevada and the other with Olympic, is striking. The complex history of hybridisation and speciation within Oregon pond populations in general (Pfrender, Spitze & Lehman, 2000) may account for this result.

One notable question that remains unanswered following our analysis is the exact structure of relationships among North American species of the *D. pulex* complex. Which group is the sister taxon to *D. melanica*? Are Nearctic *D. pulicaria* and Panarctic *D. pulex* more closely related to each other than each is to *D. melanica*? Such questions probably do not have simple answers given the complexity of relationships that exists among *D. pulex*-type species (Vergilino *et al*., 2011; Crease *et al*., 2012; Cristescu *et al*., 2012). The polytomy at the base of the clade for North American *D. pulex* species is probably not something that can be resolved with mitochondrial sequence data alone. However, sequence data from a handful of nuclear loci may not improve resolution, given that there are often striking differences among phylogenies inferred from different nuclear loci for the same set of specimens, especially those that hybridise frequently (i.e. Vergilino *et al*., 2011; Cristescu *et al*., 2012). Therefore, although the existence of four separate phylogenetic entities in North America (Nearctic *D. pulicaria*, Panarctic *D. pulex*, *D. middendorffiana* and *D. melanica*) is clearly supported by our data and the work of others (Adamowicz *et al*., 2009; Vergilino *et al*., 2011; Crease *et al*., 2012), the origin, structure and history of relationships among these entities may only be resolved, if at all, by large quantities of nuclear sequence data from throughout the genomes of each.

The melanic phenotype, once used as a diagnostic character to differentiate *D. middendorffiana* from other species such as *D. pulicaria* and *D. pulex* (Benzie, 2005), is polymorphic within three of the four major clades of North American *pulex*-type *Daphnia*. We demonstrate here that melanism occurs in some, but not all, lineages within *D. melanica*, as others have shown for Panarctic *D. pulex* (Lehman *et al*., 1995) and Nearctic *D. pulicaria* (Markova *et al*., 2007). *D. melanica* from the Sierra Nevada are melanic (Scoville & Pfrender, 2010), while closely related *D. melanica* from the Olympic Mountains are non-melanic (Miner & Kerr, 2011). Only with the advent of DNA sequence data has the protean nature of melanin pigmentation within and among species in the *D. pulex* complex become apparent.

In the case of *D. melanica* of the Olympic Mountains, we lack an obvious explanation for the absence of melanin pigmentation, which Miner & Kerr (2011) found to be minimally variable across all sampled individuals from seven populations. We must emphasise that the scale of divergence in melanin pigmentation between Sierra and Olympic populations goes beyond mere phenotypic plasticity: Sierra Nevada clones grown in the complete absence of UVR (Scoville & Pfrender, 2010) have *greater* melanin concentrations than field-collected (and therefore UVR-exposed) Olympic animals (Miner & Kerr, 2011). Why should this be? Olympic populations inhabit shallow ponds without fish predators, and many ponds have high UVR transparency (Miner & Kerr, 2011), so, selective pressures favouring photoprotective pigmentation would

seem to be present. One hypothesis advanced by Miner & Kerr (2011) concerns the difference in overall UVR intensity that results from altitudinal differences between the high-elevation Sierra Nevada and the mid-elevation Olympic Mountains, and the potential growth-rate cost of melanin pigmentation (Hessen, 1996). Under this hypothesis, any advantage of melanism in the moderate UVR habitat of the Olympic Mountains is outweighed by its growth-rate cost, whereas in the Sierra Nevada, greater UVR intensity due to higher elevation results in selection favouring melanism.

In our view, three hypotheses for the evolutionary history of pigmentation in this complex warrant consideration. The first is that the phenotype is simply evolutionarily labile and can readily be both lost and gained via a small number of genetic changes. Derived forms of *D. melanica* in Sierra Nevada habitats with introduced fish predators have evolved reduced pigmentation (Scoville & Pfrender, 2010), and the molecular pathways for melanin production are present in all populations in the ability to deposit melanin in the eyespot and ephippium. A second hypothesis, not exclusive of the first, is that the pigmentation phenotype has existed as an ancient polymorphism since before the common ancestor of today's *D. pulex* complex radiated and diversified during the Pleistocene. In this case, the presence of the polymorphism has been maintained within each lineage (e.g. *D. pulex*, *D. pulicaria*, *D. melanica*) because each inhabits a wide enough diversity of habitats that both pigmented and unpigmented forms persisted in some localities.

A third possibility is that mitochondrial phylogenies such as those presented here obscure the true evolutionary history of the lineages within the *D. pulex* complex. It is at least worth considering that the presence and absence of the pigmentation phenotype across the phylogeny of North American *pulex* group species could be entirely the result of reticulate evolution due to widespread and frequent hybridisation among lineages. From our present vantage point, however, phylogenies based on sequence data from ND5 and COI represent the most reliable and established method for describing evolutionary relationships among these lineages (Colbourne *et al*., 1998; Markova *et al*., 2007; Mergeay *et al*., 2008; Vergilino *et al*., 2011). While our results do not give definitive answers to many questions about the evolutionary history of this group, they do provide critical phylogenetic context for evaluating claims about adaptation, particularly with regard to the adaptive value of photoprotective melanin pigmentation.

Acknowledgments

Z. Baldwin, B. Dunlap, A. Coogan, E. and K. Miner, J. West, R. Ettinger, M. Rosvall and W. Palen each contributed assistance and camaraderie in the field, and L. Latta generously assisted with the collection of genetic data. This work was funded by the following National Science Foundation awards: a Doctoral Dissertation Improvement Grant (DEB-0808558) and Graduate Research Fellowship to B. E. Miner, DEB-021212487 to M. E. Pfrender, and DEB-0075509 to R.A. Knapp and O. Sarnelle. Funding was also provided to B. E. Miner by a W.T. Edmondson award from the UW Biology Department, and scholarships from Washington Trail Blazers and Washington Lake Protection Association. We thank J. Freilich and the staff of Olympic National Park for permitting and assisting our field research. Two anonymous referees provided valuable comments on an earlier draft that helped us improve the manuscript.

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(Manuscript accepted 29 March 2013)