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Divergent transcriptional responses to low temperature among populations of alpine and lowland species of New Zealand stick insects (*Micrarchus*)

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Abstract

In widespread and genetically structured populations, temperature variation may lead to among-population differentiation of thermal biology. The New Zealand stick insect genus *Micrarchus* contains four species that inhabit different thermal environments, two of which are geographically widespread. RNA-Seq and quantitative PCR were used to investigate the transcriptional responses to cold shock among lowland and alpine species to identify cold-responsive transcripts that differ between the species and to determine whether there is intraspecific geographical variation in gene expression. We also used mitochondrial DNA, nuclear 28S ribosomal DNA and transcriptome-wide SNPs to determine phylogeographic structure and the potential for differences in genetic backgrounds to contribute to variation in gene expression. RNA-Seq identified 2160 unigenes that were differentially expressed as a result of low-temperature exposure across three populations from two species (*M. hystriculeus* and *M.* nov. sp. 2), with a majority (68% ± 20%) being population specific. This extensive geographical variation is consistent across years and is likely a result of background genetic differences among populations caused by genetic drift and possibly local adaptation. Responses to cold shock shared among alpine *M.* nov. sp. 2 populations included the enrichment of cuticular structure-associated transcripts, suggesting that cuticle modification may have accompanied colonization of low-temperature alpine environments and the development of a more cold-hardy phenotype.

Keywords: adaptation, cold tolerance, cuticle, introgression, RNA-Seq

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Introduction

Insects have successfully colonized the complete range of terrestrial environments, including polar and alpine habitats where they experience subzero conditions (Sømme 1995). Subzero temperature exposure induces physiological and biochemical stresses, including increased membrane rigidity (Overgaard *et al.* 2005), induced apoptosis (Denlinger & Lee 2010), elevated oxidative stress (Lalouette *et al.* 2011) and loss of sodium ions and water from the haemolymph (MacMillan *et al.* 2012). Microarray studies on laboratory *Drosophila* populations have identified cold-responsive loci, with predicted roles in gene regulation, immune function, metabolism, stress, cuticles, membranes and the cytoskeleton (Qin *et al.* 2005; Laayouni *et al.* 2007; Sørensen *et al.* 2007; Zhang *et al.* 2011; Vesala *et al.* 2012). A majority of these candidate cold-tolerance genes in

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Drosophila do not overlap with those identified from the limited number of studies from other insects (Colinet *et al.* 2007; Purać *et al.* 2008; Clark *et al.* 2009; Teets *et al.* 2012; Dunning *et al.* 2013a). For example, the heat-shock protein *hsp83* (a homolog of mammalian *hsp90*) is up-regulated by *D. melanogaster* adults that have been exposed to 0 °C for 2 h (Qin *et al.* 2005), but not in alpine stick insects (*Micrarchus* nov. sp. 2) exposed to a similar treatment (Dunning *et al.* 2013a; 0 °C for 1 h followed by a 1-h recovery period at 20 °C). Even within *Drosophila*, patterns of cold-induced gene expression differ among species. For example, the allopatric species *D. virilis* and *D. montana* share only two consistently differentially expressed genes in response to cold acclimation (14 days at 19 °C followed by 6 days at 5 °C) and rapid cold hardening (20 days at 19 °C followed by 1 h at 0 °C; Vesala *et al.* 2012).

The thermal environment, especially the frequency, intensity and duration of low-temperature exposure, can vary considerably across the geographical range of a species (Kingsolver 1989; Chown & Terblanche 2006). As a result, populations may exhibit divergent thermal biology driven by phenotypic plasticity (Ayrinhac *et al.* 2004) or background genetic differences among populations (Sarup *et al.* 2011; Sinclair *et al.* 2012). In genetically structured populations with low rates of migration, variation in thermal stress may even promote genetic divergence and local adaptation of thermal biology (Ghalambor *et al.* 2007; Sinclair *et al.* 2012). This differentiation can be manifested at temperature-related loci in modifications to protein-coding sequences (Dahlhoff & Rank 2000) or gene expression profiles (Hoffmann & Willi 2008).

The global transcriptional responses to thermal stress have been widely studied within *Drosophila* species (Hoffmann *et al.* 2003), with much less research in other insect taxa. Microarray and RNA-Seq studies in the intertidal copepod, *Tigriopus californicus* (Schoville *et al.* 2012), and the reef-building coral, *Montastraea faveolata* (Polato *et al.* 2010), reveal strikingly divergent transcriptional responses to thermal stress among populations. In *M. faveolata*, this has been attributed to local adaptation, in spite of high gene flow among populations (Polato *et al.* 2010). Species comprised of genetically isolated populations with divergent transcriptional profiles, as a result of background genetic variation driven by drift and local adaptation, are likely to respond to climate change differently than more genetically homogeneous species (Pelini *et al.* 2009; Sinclair *et al.* 2012). Using a phylogenetic framework to investigate inter- and intraspecific variation in cold-induced transcription in closely related species and populations inhabiting different thermal environments will increase our understanding of how cold tolerance varies across the geographical range of a species and whether there is variation in this response that could be driven by divergent selection. Furthermore, shared intraspecific responses to low-temperature exposure, which differ among sister taxa, likely reflect key cold-tolerance adaptations.

We hypothesize that species with poor dispersal ability are likely to have strong phylogeographic structure as a result of genetic drift and, possibly, local adaptation. The resulting divergent genetic backgrounds are likely to contribute to variation in the intra- and interspecific transcriptional responses to environmental stress. Apterous (wingless) stick insects are therefore an excellent model to investigate whether poor dispersal leads to significant inter- and intraspecific variation in the gene expression response to low temperature. Ecological niche modelling has previously identified temperature as a determinant of the geographical distributions of two lowland New Zealand stick insect species, *Argosarchus horridus* (White) and *Clitarchus hookeri* (White), suggesting that climate has been a significant factor in driving species evolution in New Zealand phasmids (Buckley *et al.* 2009, 2010a). *Micrarchus* Carl (1913) is another endemic genus of New Zealand stick insect comprised of four apterous species that all overwinter as nymphs and/or adults (Dennis *et al.* 2014). All four species experience subzero temperatures in their respective environments, but the frequency, duration and extremes in temperature encountered by *M.* nov. sp. 2 are greater than those of the other three species (Salmon 1991; Dennis *et al.* 2014). *Micrarchus* nov. sp. 2 is exclusively found at high elevations (650–1400 m a.s.l), in contrast to lowland *M. hystriculeus*, the ecological generalist *M.* nov. sp. 1 (0–1100 m a.s.l) and *M.* nov. sp. 3 (0–283 m a.s.l; Salmon 1991; Dennis *et al.* 2014). Populations of alpine *M.* nov. sp. 2 are effectively isolated on montane 'sky islands' (Heald 1951). As *Micrarchus* species are apterous, there is limited potential for migration or gene flow among these populations. In July, alpine sites are on average 3.6 °C colder and experience ten times more freeze–thaw cycles than nearby lowland sites (Dennis *et al.* 2014). Furthermore, *M.* nov. sp. 2 has been shown to have a more cold-hardy phenotype and survives internal ice formation at a greater rate than both *M. hystriculeus* and *M.* nov. sp. 1 (A.B. Dennis, L.T. Dunning, R.D. Sinclair, R.D. Newcomb and T.R. Buckley, in prep.). Previously, RNA-Seq has been used to identify three differentially expressed genes in *M.* nov. sp. 2 from one population (Sewell Peak) after brief exposure to a mild cold shock (0 °C). These genes include an oxidoreductase enzyme, a transcriptional regulator and a cuticle protein (Dunning *et al.* 2013a). No information on the molecular response to cold is available for the other three *Micrarchus* species or further populations of *M.* nov. sp. 2.

Similarities in the transcriptional response to low-temperature exposure shared among populations of alpine stick insects, to the exclusion of lowland species, likely reflect fixed molecular mechanisms that may be associated with the colonization of the colder alpine environment. Additionally, geographically isolated populations of alpine *Micrarchus* offer an opportunity to investigate whether reduced gene flow, contributing to background genetic variation, results in differentiation of the transcriptional response to low-temperature exposure. Understanding whether species respond uniformly to temperature across their geographical range is essential to predict how species as a whole will respond to climate change-imposed selection pressures.

Materials and methods

Sample collection

Micrarchus specimens were collected across their known distributions between 2004 and 2012 (Fig. 1; full location details can be found in Table S1, Supporting information). *Micrarchus hystriculeus* (Westwood 1859) has the broadest geographical distribution (Fig. 1) and is the only representative found on the North Island (Salmon 1991). *Micrarchus* nov. sp. 1 (Voucher specimen NZAC 03000433 from New Zealand Arthropod Collection, Landcare Research, Auckland, New Zealand) is restricted to habitats on the east coast of the South Island (Salmon 1991; Dennis *et al.* 2014). *Micrarchus* nov. sp. 2 (NZAC03009458) is exclusively found at high elevations (600–1409 m above sea level) in the mountains of the northwestern South Island (Salmon 1991; Dennis *et al.* 2014; Dunning *et al.* 2013a). *Micrarchus* nov. sp. 3 (NZAC03000053) is only known from near sea level on Stephens Island (150 ha) in the Cook Strait where it is sympatric with *M. hystriculeus* (Buckley *et al.* 2012). Specimens were collected by beating and manually searching host vegetation (Salmon 1991; Dennis *et al.* 2014). Insects were preserved in ethanol or transported live to Landcare Research, Auckland.

Micrarchus phylogeny reconstruction

To assess the degree of phylogeographic structure within and among *Micrarchus* species, we collected nuclear and mitochondrial DNA sequence data. DNA was extracted from 5 to 10 mg of leg muscle tissue from 148 individuals using the Corbett X-tractor Gene robot (Corbett Robotics, Brisbane, Australia) and the QIAxtractor DX Reagents kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Mitochondrial

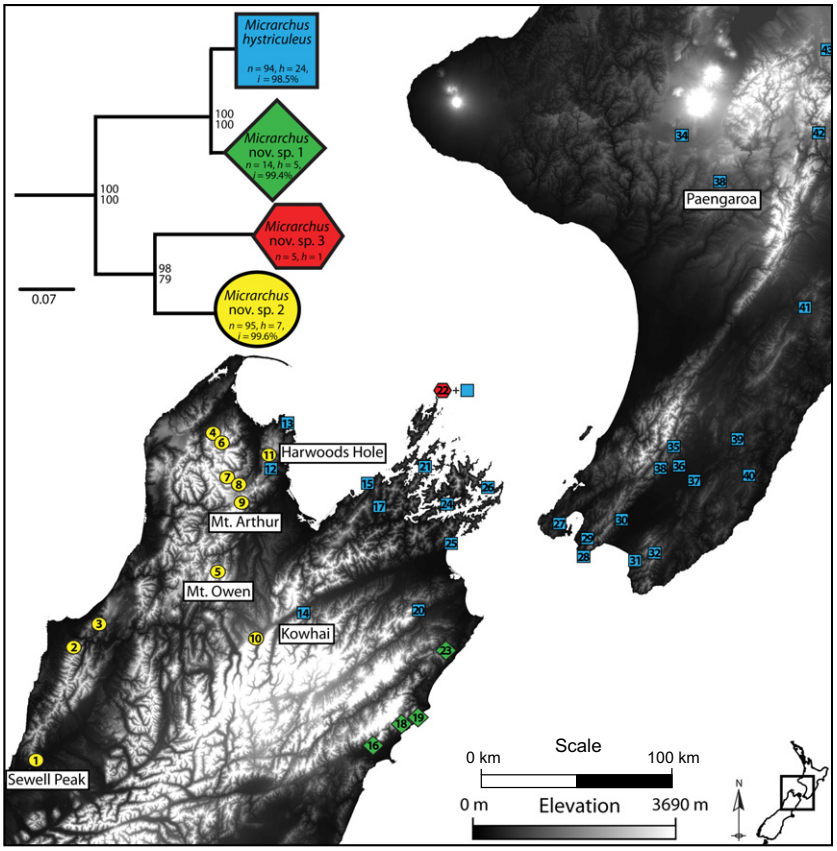

Fig. 1 Geographical distributions and phylogenetic relationships among *Micrarchus* species within New Zealand. The 28S ribosomal RNA Bayesian phylogeny is collapsed into the four species nodes, each with the number of samples (*n*), number of different haplotypes (*h*) and percentage pairwise identity between haplotypes (*i*) shown. Upper support values are posterior probabilities, and lower support values are likelihood bootstraps. Scale bar represents the number of substitutions per site. The full detail of each of the 43 sampling locations and expanded phylogeny is provided in Table S1 and Fig. S3 (Supporting information). Sites used for RNA-Seq and qPCR are named.

cytochrome oxidase subunit I (COI), cytochrome oxidase subunit II (COII) and 28S ribosomal RNA genes were sequenced using previously described methods and primers (Buckley *et al*. 2008). Sequence information for these three genes was supplemented with data from a further 36 individuals sequenced using cDNA as a template (qPCR samples) and 24 individuals using high-throughput sequencing (RNA-Seq samples). Two *Tectarchus ovobessus* and two *T. salebrosus* specimens were used as outgroups for COI/II and 28S, respectively. Both of these species are endemic to New Zealand and closely related to *Micrarchus* (Buckley *et al.* 2010b; Dunning *et al.* 2013b). Sequences were edited and assembled into alignments for each gene using GENEIOUS version 5.6.4 with default parameters (Drummond *et al.* 2012) and the MUSCLE 3.6 plug-in using default parameters, respectively. Nucleotide substitution models for phylogenetic analyses were selected based on the corrected Akaike information criterion (Sugiura 1978; Hurvich & Tsai 1989) implemented in JMODELTEST version 0.1.1 (Guindon & Gascuel 2003; Posada 2008). Bayesian phylogenies were constructed using Markov chain Monte Carlo (MCMC) sampling in MRBAYES version 3.2.0 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003), with 10 million generations sampled every thousand generations. A relative burn-in of 25% was used with the following priors: uniform substitution rates, empirically estimated state frequencies, exponential gamma shape parameter set to mean of five for among-site rate variation and proportion of invariable sites uniformly distributed between zero and one. Non-parametric bootstrap analysis under maximum likelihood (100 pseudo replicates) was performed using GARLI version 2.0 (Zwickl 2006; substitution model: COI/COII = GTR+I+Γ; 28S = TIM3 + Γ).

Cold-shock experiments

Cold-shock experiments were performed exclusively on adult females. For these experiments, *M.* nov. sp. 2 were collected from two sites: (i) Sewell Peak (SP), in the Paparoa Range, on 11 February 2011 (Site 1; Fig. 1 & Table S1, Supporting information) and; (ii) Mt Arthur (MA), Arthur Range, on 13 Feb 2011 (Site 9; Fig. 1 & Table S1, Supporting information). These two allopatric populations are separated by 176 km, much of which is comprised of low elevation forest in which this species is absent. *Micrarchus hystriculeus* was collected from Paengaroa Scenic Reserve (PA), Taihape, on 25 March 2011 (Site 38; Fig. 1 & Table S1, Supporting information). Prior to experimentation, insects were maintained under constant conditions for a minimum of 16 days of acclimatization in a 12:12 light:dark cycle under ambient room temperature and humidity, with a constant diet of freshly collected *Metrosideros excelsa* leaves. The RNA-Seq experimental design consisted of three independent trials between control and cold-shocked groups, with three biological replicates in each group for SP and PA and six biological replicates in each group for MA. Experiments were conducted on 17 March 2011 for SP and MA, and on 11 April 2011 for PA. The control groups were maintained at 21 °C in a Sanyo MIR-154 incubator (Global Science and Technology, Osaka, Japan) prior to being snap-frozen in liquid nitrogen. The 21 °C temperature used for the control group is lower than the maximum temperature experienced by *M.* nov. sp. 2 in the wild (27.40 °C ± 1.28 °C; Dennis *et al.* 2014). The cold-shocked experimental individuals were incubated at 21 °C for 1 h prior to cooling at approximately 1 °C per minute until the incubator reached −5 °C, where it was held for 1 h. The animals were then warmed at approximately 1 °C per minute and held at 21 °C for a 1 h recovery, at which point insects were snap-frozen and stored at −80 °C prior to RNA extraction. The selected cold-shock temperature is close to the minimum temperature experienced by *M.* nov. sp. 2 in the wild (−4.86 °C ± 1.06 °C; Dennis *et al.* 2014). All insects survived the treatments and were moving in a coordinated fashion prior to snap-freezing.

cDNA preparation and RNA-Seq

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) from the head, antennae and prothorax of the 24 experimental samples using previously published methods (Dunning *et al.* 2013a). The 24 cDNA libraries for high-throughput sequencing (HTS) were prepared using the Illumina TruSeq RNA Sample Preparation Kit (Illumina San Diego, CA, USA) according to the manufacturer's protocol, with a starting input of 2.65 μg total RNA from each sample. Individual samples had randomly assigned barcodes ligated to the cDNA fragments and were amplified with 13 cycles of PCR. Libraries were validated using the Agilent 2100 Bioanalyzer to ensure each had the recommended ~260 bp average fragment size. Libraries were sent for HTS at the University of Utah's Microarray and Genomic Analysis Shared Resource where they were quantitative RT–PCR (qPCR)-quantified prior to sequencing on three lanes of a 50 cycle single-end Illumina HiSeq 2000 run.

Pre-processing, assembly and annotating RNA-Seq data

Reads with ambiguous bases were removed using SHORT READ version 1.16.3 (Morgan *et al.* 2009) implemented in R version 2.15.0 (R Development Core Team 2012). Illumina adapter sequences and low-quality bases

(Phred quality < 30) were trimmed using CUTADAPT version 1.1 (Martin 2011). Poly A/T tails longer than 10 bp from either end of the reads, and reads shorter than 40 bp were removed using PRINSEQ LITE version 0.16 (Schmieder & Edwards 2011). Cleaned reads were grouped by population and de novo assembled using Trinity (release 2012-10-05, Grabherr *et al.* 2011), with default parameters and the *reduce* option in Butterfly to combine similar splice variants. Ribosomal RNA was removed using all available databases with RIBOPICKER version 0.4.3 (Schmieder *et al.* 2012) and BLASTn sequence searching against 28S *Micrarchus* sequences. Assemblies were annotated by BLASTx (*E*-value threshold = $1e^{-10}$) sequence searching against the National Centre for Biotechnology Information (NCBI) non-redundant (*nr*) protein and SwissProt databases. Gene Ontology (GO) annotation was based on the SwissProt Blast matches using BLAST2GO version 2.6.2 (Conesa *et al.* 2005). GO annotations were compared and visualized between assemblies using WEGO (Ye *et al.* 2006).

Differential expression analysis of RNA-Seq data

Individual cleaned sequence reads were mapped back onto the respective population Trinity assemblies using RSEM version 1.2.4 (Li & Dewey 2011). To be considered for differential expression analysis, 'unigenes' (Trinity components containing clusters of 'contigs' representing splice variants of the same locus) had to have at least one count per million in half of the samples analysed (three of six in PA and SP; six of 12 in MA). Differential expression analysis followed previously published methods (Dunning *et al.* 2013a). In brief, the DESEQ version 1.12.0 (Anders & Huber 2010), EDGER version 3.2.4 (Robinson *et al.* 2010) and BAYSEQ version 1.14.1 (Hardcastle & Kelly 2010) packages implemented in R version 3.0.1 were used to determine which genes were differentially expressed whilst controlling for Type I errors (false discovery rate (FDR) <0.05 for edgeR, and <0.10 for DESeq and baySeq). Genes were considered as differentially expressed if they were significant for at least one of the differential expression analyses. Gene Ontology annotation enrichment analysis was performed by way of a Fisher's exact test in Blast2GO (Conesa *et al.* 2005) between the GO terms associated with the differentially regulated cold-responsive unigenes and those associated with the non-differentially expressed unigenes. The analysis was restricted to biological function with a FDR of <0.10.

qPCR validation

To validate the RNA-Seq results and gain more detailed information on the variation in expression among populations and species, a subset of six genes, possessing a range of GO terms and direction of change in gene regulation (Table S2, Supporting Information), was selected for qPCR analysis using both technical and biological replicates. Two non-differentially expressed reference genes across all three comparisons in the RNA-Seq data were selected to normalize the relative qPCR expression between genes (*pyruvate kinase* and *ATP synthase subunit beta*; $P > 0.85$; counts > 1000). Technical replicates used cDNA synthesized from the same individuals as RNA extractions used for the RNA-Seq data. Biological replicates consisted of cDNA synthesized from different individuals from the same population, with *M.* nov. sp. 2 individuals collected and treated on different dates to the technical replicates; SP collected on 27 January 2012 and treated on 28 February 2012; MA collected on 23 January 2012 and treated on 2 March 2012. In addition, three further populations were used for qPCR. These were *M.* nov. sp. 2 collected from: (i) Mt. Owen (MO), Nelson Range on 25 January 2012 and treated on 1 March 2012 (Site 5; Fig. 1 & Table S1, Supporting Information); and (ii) Harwood's Hole (HH), Takaka Hill on 24 January 2012 and treated on 29 February 2012 (Site 11; Fig. 1 & Table S1, Supporting information). *Micrarchus hystriculeus* was collected from Kowhai (KO), Wairau Valley, on 19 January 2012 and treated on 25 April 2012 (Site 14; Fig. 1 & Table S1, Supporting information). For biological and population replicates, experimental treatments and RNA extractions followed the methods described above for the RNA-Seq samples. Contaminating DNA was removed from RNA extractions prior to cDNA synthesis using TURBO DNase (Invitrogen, Carlsbad, CA). Primer design (primer sequences Table S3, Supporting information), cDNA library preparation and qPCR followed previously described methods (Dunning *et al.* 2013a). Fold changes were generated by dividing the mean relative amount for each treatment group by the mean relative amount of the control. Linear regression with Pearson correlation coefficients (r) was used to compare the agreement between the RNA-Seq and qPCR results. For the additional HH, KO and MO populations, significant differential expression between treatments was assessed using the log-transformed raw relative expression values and a one tailed *t*-test.

Population genetics and phylogeography of Micrarchus

Contigs corresponding to all 13 mitochondrial protein-coding genes were extracted from the three RNA-Seq assemblies by tBLASTx sequence searching against the complete *D. melanogaster* mitochondrial genome (DMU37541). The contigs were subsequently aligned and pairwise nucleotide similarities calculated. SNP data

were generated from a new Trinity assembly constructed with the cleaned reads from all 24 individuals with the same parameters as previously described. Individual clean reads were then mapped onto the new combined assembly using Bowtie 2 (Langmead & Salzberg 2012). SNPs were subsequently called from the Bowtie 2 mapped reads using the recommended default parameters for *mpileup* in SAMTOOLS version 0.1.18 (Li *et al.* 2009). The data set was trimmed to only retain biallelic, unlinked and high-quality (Q > 30) SNPs with no missing data using VCFTOOLS version 0.1.10 (Danecek *et al.* 2011). Population structure was assessed by way of a principle component analysis (PCA) performed in R and ancestry inference using ADMIXTURE version 1.22 (Alexander *et al.* 2009) with $K = 2–6$.

Results

Transcriptome assembly

A total of 235 million 50-bp single-end Illumina reads were obtained from 24 individually tagged cDNA libraries. Raw data have been submitted to the NCBI Sequence Read Archive (SRA) with accession numbers SRX395469, SRX395470 and SRX395471. An average of 9.8 million reads (range: 6.6–13.2 million reads) were generated for each library (Table S4, Supporting information), with 99% of the data remaining after trimming. Cleaned reads were de novo assembled by species and population: SP (*Micrarchus* nov. sp. 2), MA (*M.* nov. sp. 2) and PA (*M. hystriculeus*), producing 42 425 (41 771 unigenes), 58 743 (57 576 unigenes) and 37 814 (37 334 unigenes) contigs, respectively (Table S4, Supporting information). Between 27% and 30% of the contigs within each assembly were annotated by BLASTx against the *nr* database, with the species distribution of top matches overlapping among the three assemblies (Fig. S1, Supporting information). The distribution of high level GO terms from the SwissProt BLASTx matches was highly similar among the three assemblies (Fig. S2, Supporting information).

Phylogeography and population genetics

Sequence data were obtained from 210 individuals, resulting in alignments of 530 bp for 28S and 1515 bp of COI/COII. All sequences have been submitted to NCBI GenBank (accession numbers KJ142327 to KJ142746). The 28S Bayesian and likelihood phylogenies support each *Micrarchus* species as monophyletic, with *M. hystriculeus* sister to *M.* nov. sp. 1 and *M.* nov. sp. 2 sister to *M.* nov. sp. 3 (Fig. 1; expanded phylogeny Fig. S3, Supporting information). There is very little intraspecific variation at this locus, with between 98.5% and 100% pairwise similarity between alleles from the same species. The COI/COII Bayesian phylogeny does not support any *Micrarchus* species as monophyletic, with geographically proximal populations of different species often grouping together (Fig. 2; expanded phylogeny Fig. S4, Supporting information). For example, the *M.* nov. sp. 2 haplotype clade containing SP, Denniston

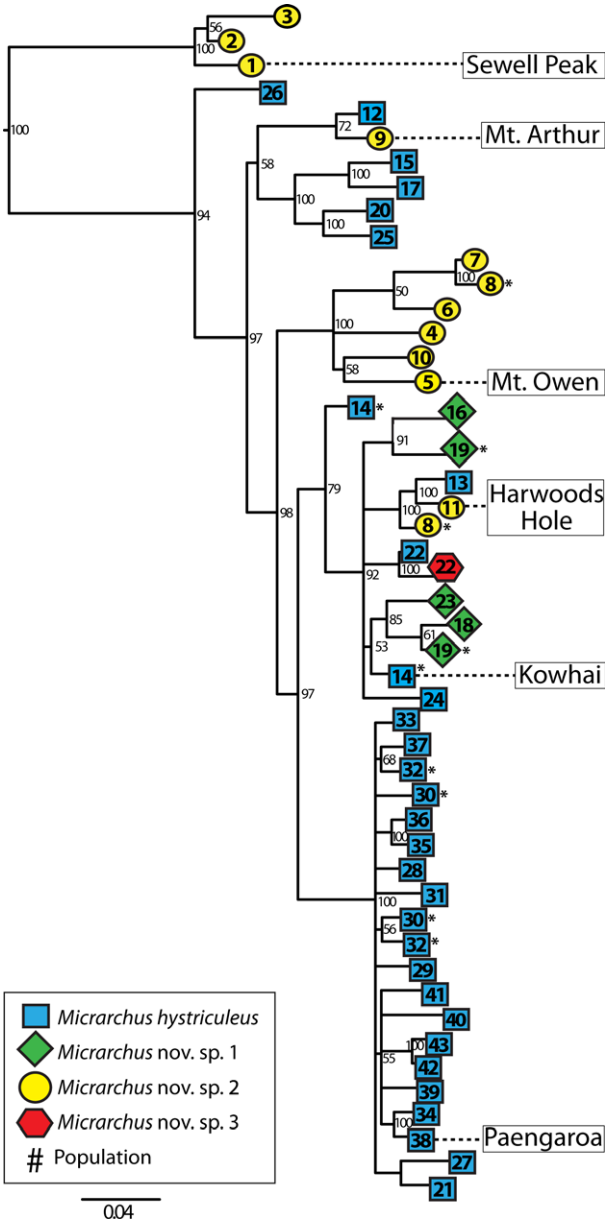

Fig. 2 Bayesian phylogeny of *Micrarchus* species constructed using mitochondrial cytochrome oxidase subunit I (COI) and II (COII) DNA sequences representing 208 individuals from 43 populations. Nodes were collapsed to population, with those that are non-monophyletic denoted with an asterisk. Support values are posterior probabilities. Scale bar represents the number of substitutions per site. Sites used for RNA-Seq and qPCR are named.

Plateau and Buckland's Peak is sister to all other *Micrarchus* haplotypes, separated by the longest branch in the ingroup phylogeny. The other clades containing *M.* nov. sp. 1, *M.* nov. sp. 2 and *M.* nov. sp. 3 are nested within clades of *M. hystriculeus*. *Micrarchus* nov. sp. 3 is endemic to Stephens Island (location 22; Fig. 1), where it is sympatric with the widespread *M. hystriculeus*. Whilst distinct at the 28S locus, *M.* nov. sp. 3 shares mtDNA with *M. hystriculeus* from Stephens Island.

All MA (*M.* nov. sp. 2) mtDNA protein-coding sequences in the RNA-Seq data have a greater pairwise nucleotide identity to those from PA (*M. hystriculeus*; average pairwise identity = 95.2%) than the conspecific SP population (average pairwise identity = 92.7%), apart from *NADH dehydrogenase subunit 4L* (*ND4L*). The *ND4L* gene is 285 bp in MA is 98.1% identical to PA (11 observed substitutions) and 98.6% identical to SP (ten observed substitutions). The degree of admixture in the nuclear genome between *M.* nov. sp. 2 and *M. hystriculeus* was assessed using 45 784 biallelic unlinked transcriptome-wide SNPs extracted from all 24 individuals from three populations: 6 × PA (pure *M. hystriculeus* mtDNA); 6 × SP alpine *Micrarchus* (pure *M.* nov. sp. 2 mtDNA); and 12 × MA alpine *Micrarchus* (introgressed *M. hystriculeus* mtDNA). PCA analysis of the SNP data identified three separate clusters that corresponded to the three populations (Fig. 3). The first principle component explained 26.2% of the variation in the data and separates both *M.* nov. sp. 2 populations from *M. hystriculeus*. The second principle component explained 14.6% of the variation and distinguishes the two *M.* nov. sp. 2 populations.

Maximum likelihood estimation was used to determine the ancestry of each individual from the SNP data, with three being the optimal number of ancestral populations based on cross-validation errors between different values of *K* (2–6). *K* = 3 clearly separates the three populations (Fig. 3). The next best fit of *K* = 2 groups the populations into their respective species groups. Values of K higher than three have a much poorer fit to the data (higher cross-validation error). These analyses demonstrate a high degree of differentiation in the nuclear genomes of the species of *Micrarchus*; however, there is strong evidence for gene flow within the mtDNA.

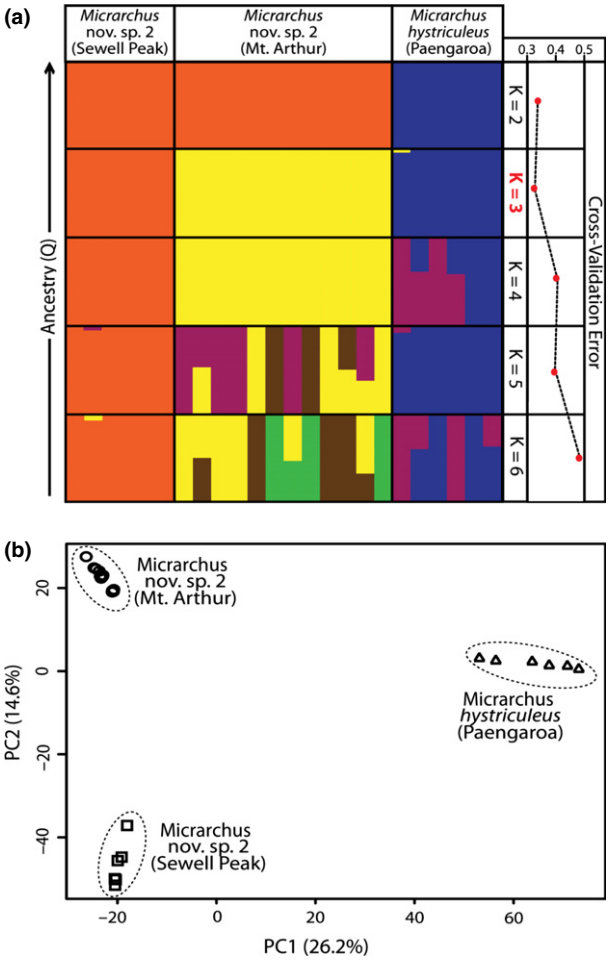

Fig. 3 (a) Inferred ancestry (Q-Plot) and (b) principle components analysis based on 45 784 biallelic unlinked SNPs from 24 individuals representing three populations of two *Micrarchus* species (Paengaroa = *M. hystriculeus*; Mt. Arthur and Sewell Peak = *M.* nov. sp. 2). Each individual in the Q-plot is represented by one vertical bar divided into varying proportions (colours) representing ancestral populations (*K*). The optimal value of *K* is determined by the lowest cross-validation procedure score (*K* = 3).

Differential expression analysis

Differentially expressed transcripts as a result of cold-shock treatment were identified in MA, SP and PA *Micrarchus* populations by comparing control and cold-shock individuals in each population separately. The cold-responsive differentially expressed loci were then compared between populations and species. Counts for differential expression analysis were generated by mapping cleaned 50 bp Illumina HiSeq reads back onto their respective population transcriptome, with between 84.3% and 92.9% (Mean = 89.5%; SD = 1.9%) of the reads from each individual having at least one valid alignment. Differential expression analyses identified 1774, 252 and 134 differentially expressed cold-responsive unigenes from SP, MA and PA *Micrarchus* populations, respectively (Table 1; Full details of the differentially regulated cold-responsive genes in Supplementary File B). A majority of the differentially expressed unigenes were unique to each population (MA 52%; SP 90%; PA 62%; Mean 68% ± 20%), sharing no BLASTx match (*E*-value threshold ≤ $1e^{-10}$) with differentially expressed genes

Table 1 Differentially expressed cold-shock responsive transcripts in *Micrarchus* stick insects identified using RNA-Seq. Cold-shock-treated individuals were compared to controls using three exact test approaches (edgeR with common dispersion, edgeR with tagwise dispersion and DESeq) and a Bayesian method (baySeq)

Species	*Micrarchus* nov. sp. 2				*Micrarchus hystriculeus*	
Population	Sewell Peak		Mt. Arthur		Paengaroa	
Unigenes*	24 629		22 482		23 151	
Direction	Up	Down	Up	Down	Up	Down
edgeR†	173	1077	60	165	81	37
edgeR‡	54	900	0	0	23	9
DESeq	40	275	0	2	5	1
baySeq	279	1070	20	24	29	6
Total	424	1350	78	174	94	40

Significance cut-off for: edgeR = false discovery rate (FDR) < 0.05 ($P < 0.003$); DESeq = FDR < 0.10 ($P < 0.002$); baySeq FDR < 0.10 (likelihood > 0.75).
Total = unique unigenes called by all analysis methods.
*Number of unigenes used for differential expression analysis after removing unigenes with less than one count per million in at least three samples for Sewell Peak/Paengaroa and six samples for Mt. Arthur.
†Using common dispersion.
‡Using tagwise dispersion.

identified in other populations (Fig. 4). Cold-responsive unigenes that did overlap between populations were regulated in similar and opposing directions (Fig. 4; Full details of correspondence between differentially regulated cold-responsive transcripts in Table S5, Supporting information). No unigene was universally up-regulated or universally down-regulated as a result of cold shock in all three populations.

Enrichment of biological function GO terms in the differentially expressed unigenes compared with the non-differentially expressed unigenes was assessed using a Fisher's exact test (FDR < 0.05). This identified 32 molecular functions in SP, three in MA and one in PA (Table 2). The GO terms for the structural constituent of cuticle (GO:0042302), structural constituent of chitin-based cuticle (GO:0005214) and structural molecule activity (GO:0005198) were significantly enriched in both MA and SP *M.* nov. sp. 2 populations.

Quantitative PCR

To validate the RNA-Seq results and to further explore geographical patterns of differential expression, transcript levels were also measured in a subset of six genes (Table S2, Supporting information) by qPCR using both technical (same individuals and RNA extractions for both methods) and biological replicates (different individuals from the same population) in all three populations. Overall, there was a strong correlation between the RNA-Seq and qPCR results for the technical

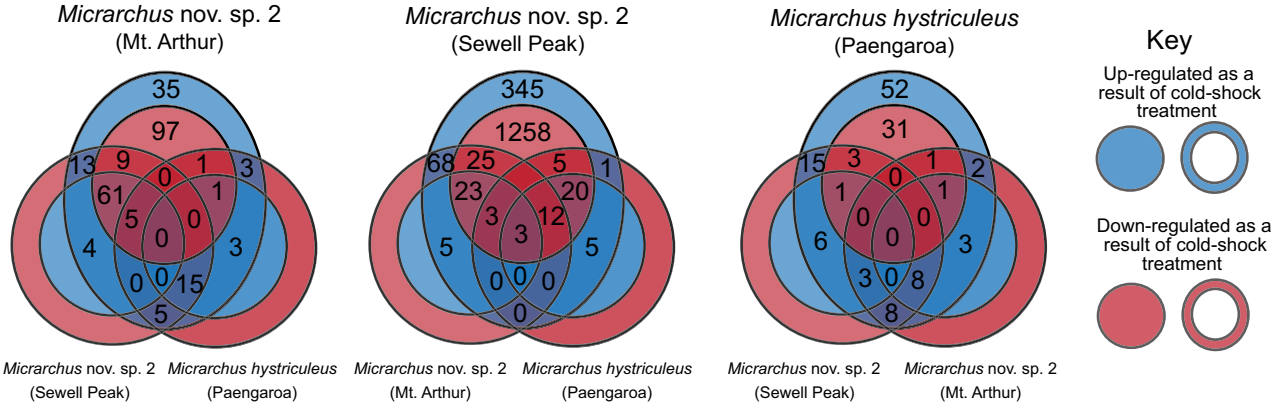

Fig. 4 Intra- and interspecific variation in differentially expressed cold-responsive loci from three populations representing two species of *Micrarchus* stick insect. Each Venn diagram represents a population and the number of its differentially expressed unigenes that have a BLASTx ($E < 1^{-10}$) sequence match with a differentially expressed unigenes in the other populations.

Table 2 Enriched molecular function Gene Ontologies (GO) as a result of cold-shock treatment in two species of *Micrarchus* stick insects. GO terms from unigenes identified as differentially expressed by either edgeR, DESeq or baySeq analysis were compared to GO terms for non-differentially expressed unigenes to assess enrichment of molecular function using a Fishers exact test

GO term	Definition	FDR	*P*	#DRR	#CS	#CO	#GO
Micrarchus nov. sp. 2 (Sewell Peak)							
GO:0042302	Structural constituent of cuticle	6.12E-07	1.33E-10	12	12	0	23
GO:0005214	Structural constituent of chitin-based cuticle	6.21E-04	8.56E-07	7	7	0	13
GO:0030228	Lipoprotein particle receptor activity	1.56E-03	2.83E-06	11	1	10	41
GO:0038024	Cargo receptor activity	2.70E-03	9.58E-06	11	1	10	46
GO:0022843	Voltage-gated cation channel activity	3.03E-03	1.14E-05	8	1	7	24
GO:0005041	Low-density lipoprotein receptor activity	4.25E-03	1.95E-05	7	1	7	19
GO:0005245	Voltage-gated calcium channel activity	5.09E-03	2.87E-05	6	0	6	14
GO:0043924	Suramin binding	5.09E-03	2.96E-05	5	0	5	9
GO:0005244	Voltage-gated ion channel activity	7.66E-03	5.36E-05	8	1	7	29
GO:0022832	Voltage-gated channel activity	7.66E-03	5.36E-05	8	1	7	29
GO:0005219	Ryanodine-sensitive calcium-release channel activity	7.66E-03	5.68E-05	5	0	5	10
GO:0034236	Protein kinase A catalytic subunit binding	7.66E-03	5.68E-05	5	0	5	10
GO:0005200	Structural constituent of cytoskeleton	1.21E-02	1.09E-04	14	1	13	90
GO:0004043	L-aminoadipate-semialdehyde dehydrogenase activity	1.30E-02	1.17E-04	3	3	0	3
GO:0034237	Protein kinase A regulatory subunit binding	1.70E-02	1.64E-04	5	0	5	12
GO:0015026	Coreceptor activity	1.76E-02	1.77E-04	4	0	4	7
GO:0001948	Glycoprotein binding	2.28E-02	2.46E-04	10	1	9	54
GO:0002162	Dystroglycan binding	2.35E-02	2.57E-04	5	0	5	13
GO:0008092	Cytoskeletal protein binding	2.70E-02	3.18E-04	40	7	33	466
GO:0051018	Protein kinase A binding	3.12E-02	3.83E-04	5	0	5	14
GO:0071936	Coreceptor activity involved in Wnt receptor signalling pathway	3.37E-02	4.52E-04	3	0	3	4
GO:0070016	Armadillo repeat domain binding	3.37E-02	4.52E-04	3	0	3	4
GO:0008802	Betaine-aldehyde dehydrogenase activity	3.37E-02	4.52E-04	3	3	0	4
GO:0008010	Structural constituent of chitin-based larval cuticle	3.37E-02	4.52E-04	3	3	0	4
GO:0005198	Structural molecule activity	3.37E-02	4.53E-04	34	13	21	381
GO:0005218	Intracellular ligand-gated calcium channel activity	3.92E-02	5.52E-04	5	0	5	15
GO:0034189	Very-low-density lipoprotein particle binding	4.04E-02	5.90E-04	4	0	4	9
GO:0002020	Protease binding	4.04E-02	5.99E-04	8	0	8	40
GO:0034185	Apolipoprotein binding	5.23E-02	8.43E-04	8	0	8	42
GO:0030492	Haemoglobin binding	6.22E-02	1.05E-03	5	0	5	17
GO:0042954	Lipoprotein transporter activity	7.86E-02	1.39E-03	5	0	5	18
GO:0008013	Beta-catenin binding	9.59E-02	1.85E-03	7	0	7	37
Micrarchus nov. sp. 2 (Mt. Arthur)							
GO:0042302	Structural constituent of cuticle	1.84E-14	1.25E-18	9	2	7	16
GO:0005214	Structural constituent of chitin-based cuticle	1.74E-06	2.37E-10	5	2	3	10
GO:0005198	Structural molecule activity	6.28E-02	1.28E-05	9	2	7	367
Micrarchus hystriculeus (Paengaroa)							
GO:0047714	Galactolipase activity	7.89E-02	1.14E-05	2	0	2	3

GO, gene ontology; FDR, false discovery rate; #DR, Number differentially regulated; #CS, Number of differentially regulated unigenes with increased expression in cold-shock treatment group; #CO, Number of differentially regulated unigenes with increased expression in control group; #GO, total number in category.

replicates ($r = 0.922$, $P < 0.001$; Fig. 5; Fig. S5, Supporting information). There was also a significant positive correlation between the RNA-Seq and qPCR results for the biological replicates ($r = 0.573$, $P = 0.016$; Fig. 5, Fig. S5, Supporting information). To obtain further information on intraspecific patterns of gene expression, the expression of the qPCR candidates was also assessed in MO and HH *M.* nov. sp. 2 and KO *M. hystriculeus* populations (Fig. 5). There was a high level of intra- and interspecific variation in the magnitude of expression response to the cold-shock treatment. For example, in *Endocuticle structural glycoprotein db-2* (*Cud2*), the direction of differential regulation in the additional alpine *Micrarchus* populations was consistent with MA, being significantly down-regulated as a result of cold shock in HH. *Sarcosine dehydrogenase* (*Sardh*) was significantly up-regulated in HH, but not in MO, even though the overall fold change was higher in the latter. *Cathepsin L* was

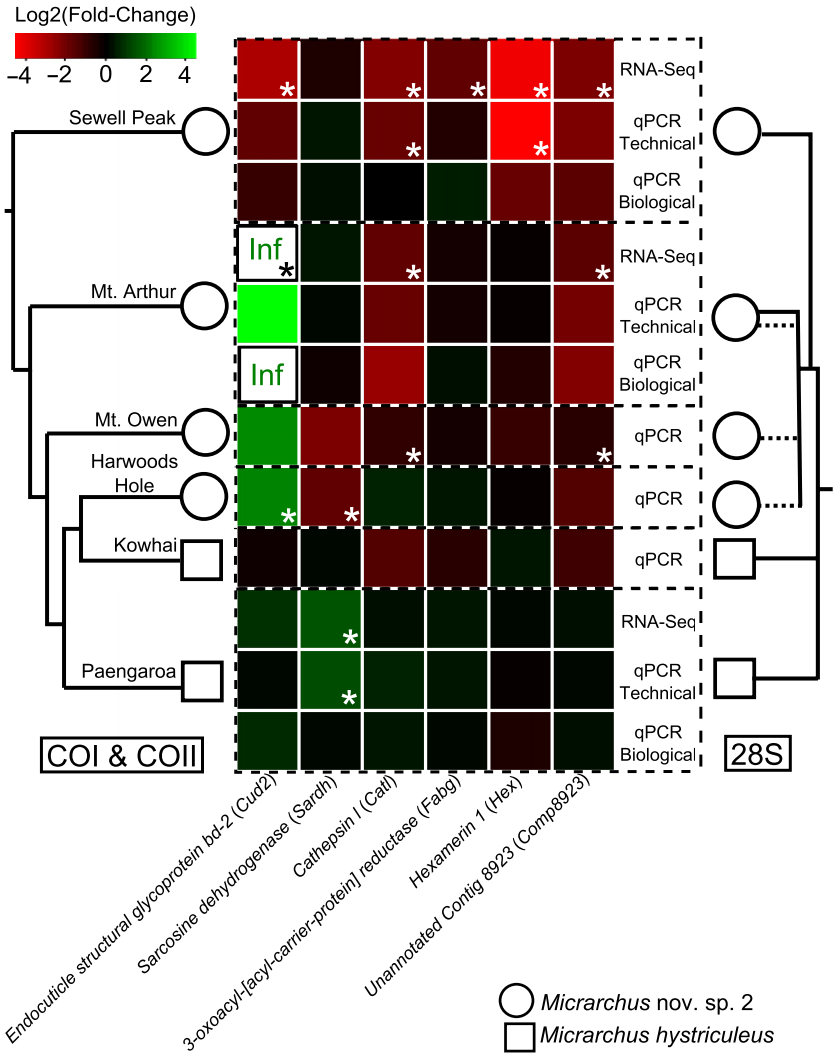

Fig. 5 Heat map representing log-transformed fold changes in gene expression between control and cold-shocked *Micrarchus* stick insects using RNA-Seq and qPCR. Asterisks indicate significantly differentially expressed genes (qPCR samples *t*-test $P < 0.05$; RNA-Seq samples false discovery rate < 0.05 edgeR or < 0.10 baySeq/DESeq). COI/COII, and 28S *Micrarchus* phylogenies represented by cartoons, with the dotted line representing shared haplotypes. Negative fold changes represent increased expression in treatment group, and positive fold changes represent increased expression in control group. Two samples have infinite fold change (Inf) as expression was recorded in only one treatment group.

significantly up-regulated in three of the four *M.* nov. sp. 2 populations (MA, SP and MO).

Discussion

All *Micrarchus* experience subzero temperatures in their respective environments. However, the frequency and duration of cold stress, and the minimum temperatures encountered by *M.* nov. sp. 2 are more extreme than those experienced by its congeners (Salmon 1991; Dennis *et al.* 2014). Furthermore, *M.* nov. sp. 2 is known to survive internal ice formation at a greater rate than both *M. hystriculeus* and *M.* nov. sp. 1 (A.B. Dennis, L.T. Dunning, R.D. Sinclair, R.D. Newcomb and T.R. Buckley, in prep.). Thus, we expected interspecific differences in the transcriptional response to low-temperature exposure to have evolved between the species. In this study, we show that genes associated with structural reorganization of the cuticle in other species appear to be an evolutionarily conserved response to low-temperature exposure across all populations of cold-hardy alpine *M.* nov. sp. 2. This suggests that modifications to the cuticle structure may play an important role in conferring increased cold tolerance in this alpine species.

Genetic divergence and introgression among Micrarchus species and populations

Our phylogeographic data clearly show strong differentiation of the nuclear genomes of *Micrarchus* species and populations within *M.* nov. sp. 2. All species are monophyletic at 28S, and transcriptome-wide SNPs are clearly able to discriminate populations and species. However, this resolution is lost in the mitochondrial genome, with evidence of introgression of *M. hystriculeus* mtDNA into the other three *Micrarchus* species (Fig. 2). The consistent phylogenetic clustering of geographically proximate

populations from different *Micrarchus* species supports our conclusion that mtDNA replacement is due to introgression rather than incomplete lineage sorting (Holder *et al.* 2001). Furthermore, introgression is commonly observed in stick insects, including New Zealand species (Morgan-Richards & Trewick 2005; Andersen *et al.* 2006; Buckley *et al.* 2008; Schwander & Crespi 2009). Several processes in insects may give rise to the complete mitochondrial capture with reduced nuclear introgression that is witnessed in *M.* nov. sp. 2 from Mount Arthur (reviewed by Toews & Brelsford 2012), including (i) hybrid zone movement, (ii) sex-biased asymmetries, (iii) adaptive introgression, (iv) demographic disparities and (v) *Wolbachia* infection. However, further work is required to identify the cause of complete mitochondrial capture in some *Micrarchus* populations.

Cold-induced changes to transcription in Micrarchus

The de novo assembled transcriptomes of the three *Micrarchus* populations were broadly similar with analogous GO annotation and BLAST match frequencies within and between species (Figs S1 & S2, Supporting information). However, extensive variation in the transcriptional response to cold shock mirrors the genetic diversity witnessed in *Micrarchus*, with 68% (±20%) of significantly differentially expressed unigenes being location specific. The most notable difference in transcriptional response between these two species is the differential expression of cuticle-related unigenes in *M.* nov. sp. 2, but not in *M. hystriculeus*. In the two genetically distinct and geographically isolated populations of alpine *M.* nov. sp. 2 used for RNA-Seq, structural cuticle unigenes are predominately differentially regulated in opposite directions. This dichotomy in expression response may be part of a complex time-series of reaction, as seen during insect moulting (Riddiford 1981), with the speed of reaction in the transcriptional response differing among populations. Geographically isolated populations of marine copepods show similar disparate responses to thermal stress in genes associated with cuticle structure that are attributed to local thermal adaptation (Schoville *et al.* 2012). Structural reorganization of the cuticle in response to cold shock in *M.* nov. sp. 2 may be an adaptation that has facilitated the colonization of the alpine environment and the development of a more cold-hardy phenotype.

At the individual cuticle unigene level, both MA and SP *M.* nov. sp. 2 populations up-regulate two *cathepsin L* (*Catl*) paralogues as a result of cold shock. *Catl* is a proteolysis enzyme acting on chitin-based cuticles that is also up-regulated in Arctic springtails during cryoprotective dehydration (Clark *et al.* 2009). We previously identified a cold-responsive cuticular protein up-regulated by *M.* nov. sp. 2 in response to a mild cold shock (1 h 0 °C with 1 h recovery at 20 °C; Dunning *et al.* 2013a). However, this gene (*cuticular protein analogous to peritrophins 3-d2*) was not differentially expressed in the current study, illustrating variation in the transcriptional response to mild and severe cold-shock consistent with other physiological differences between different intensities of cold exposure (reviewed by Sinclair & Roberts 2005). Cold-responsive cuticular genes and proteins have been identified in many other insect species including flies (Qin *et al.* 2005), wasps (Colinet *et al.* 2007), beetles (Carrasco *et al.* 2011) and locusts (Wang *et al.* 2012), indicating that changes in this tissue likely play an important role in adaptation to low temperature. However, the physiological role of cuticle-related proteins in insect cold tolerance (and indeed, whether their primary role is in the cuticle) has not yet been determined.

The evolution of gene expression in a phylogenetic context

Structural reorganization of the cuticle appears to be an evolutionarily conserved response to low-temperature exposure across all populations of cold-hardy *M.* nov. sp. 2. However, a majority of the other differentially expressed unigenes are population specific. For example, three environmental stress response *cytochrome P450* unigenes (*Cp6k1*, *c4 g15* and *Cpgj1*; Sang *et al.* 2012) and an insect cryoprotectant-synthesis enzyme, *Aldose reductase* (*Aldr*; Koštál *et al.* 2004), are significantly up-regulated in SP, but not in any of the other populations or species. The high proportion of population-specific responses could result from phenotypic plasticity or background genetic variation resulting from genetic drift and/or local adaptation – or a combination of all three of these processes. We acclimated summer wild-caught insects under standard laboratory conditions for at least 16 days prior to cold-shock experiments in an effort to compensate for any *in situ* cold hardening and therefore minimizing phenotypically plastic responses. In addition, population-specific *M.* nov. sp. 2 responses are consistent among biological replicates collected from the wild in February 2011 and January 2012. Although we have only 1 year of microclimate data, supplementary data are available from National Institute of Water and Atmospheric Research (NIWA) weather station located in a similar alpine habitat between MA and SP [Mahanga electronic weather station; −42 °01.066′, 171°20.585′; 1995 m a.s.l, CliFlo database (cliflo.niwa.co.nz)]. At Mahanga, the winter months (June to August) prior to the 2010 collection were wetter (total rainfall 2010: 301 mm) than in 2011 (172 mm), and 2010 had colder mean (2010: −1.9 °C ± 2.3; 2011: 0.6 °C ± 3.4), daily maximum (2010: 1.7 °C ± 3.2; 2011: 3.8 °C ± 4.1) and daily

minimum (2010: −4.3 °C ± 2.3; 2011: −1.9 °C ± 3.5) temperatures than 2011. These very different thermal environments suggest that developmental-induced phenotypic plasticity in *M.* nov. sp. 2 is unlikely to underpin intraspecific variation in transcriptional response to low temperature, as there is annual variation in temperatures individuals encounter during overwintering and maturation.

A more likely explanation for the intraspecific variation in transcriptional response to low-temperature exposure is that the response is governed by differences in background genetic variation among populations. The montane 'sky islands' distribution of wingless *M.* nov. sp. 2, and the resulting reduced migration between populations, could result in increased levels of genetic drift with regard to transcriptional variation (Whitehead & Crawford 2006). The small population sizes, geographical temperature variation and extremely low migration rates could potentially increase the efficacy of selection on variation in expression response to low-temperature exposure. Combined, these factors increase the chance of fixation of locally adapted traits that have even relatively small positive selective coefficients (Lenormand 2002; Kawecki & Ebert 2004). Given that the differential regulatory responses we have observed are in response to a significant abiotic stressor, it is likely that a proportion of this variation is adaptive. However, without additional populations and reciprocal transplants to determine the extent of local adaptation, we are currently unable to discriminate between drift and selection in driving the variation in gene expression that we have observed.

Whilst the 'core' transcriptional response of cuticular gene expression has been maintained in all the montane populations, the variation in low-temperature response may mean that different populations of alpine *M.* nov. sp. 2 will respond differently to future altered climate regimes. This is contrary to genetically homogenous species, where high dispersal between populations may prevent the maintenance of local alleles and lead to a more uniform gene expression response (Case & Taper 2000). Furthermore, these results suggest that divergent expression responses to abiotic stress may evolve rapidly among closely related populations and species. Currently, there is very little information regarding population-level variation in the transcriptional responses to abiotic stressors. However, it appears likely that genetically structured populations with reduced gene flow will show some differentiation in their transcriptional profiles as a result of their divergent genetic backgrounds, although it remains to be determined whether or not this divergence is adaptive at present. Nevertheless, such populations have the potential to show divergent evolutionary trajectories as they evolve in response to future temperature challenges.

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Data accessibility

Nucleotide sequences have been submitted to the National Center for Biotechnology Information (NCBI) GenBank database (accession numbers KJ142327 to KJ142746) and raw Illumina RNA-Seq data has been submitted to NCBI Sequence Read Archive (SRA; accession numbers SRX395469, SRX395470 and SRX395471). Annotated assemblies, differential expression data/results, raw qPCR data, SNP data, mtDNA and 28S ribosomal DNA sequence alignments and tree files are available on Dryad: doi:10.5061/dryad.6p5t3.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 *Micrarchus* sampling localities.

Table S2 Target genes used for qPCR to validate RNA-Seq expression estimates exhibit a range of biological function

Gene ontologies (GO)

Table S3 qPCR primer sequences; primer name denotes direction (F= forward; R = reverse).

Table S4 Summary statistics for *Micrarchus* stick insect de novo transcriptome assemblies generated from 50 bp single-end Illumina HiSeq RNA-Seq data.

Table S5(a-c) Intra- and interspecific overlap in differentially expressed cold-responsive loci from three populations representing two species of *Micrarchus* stick insect.

Table S5b *Micrarchus* nov. sp. 2 from Mt. Arthur.

Table S5c *Micrarchus hystriculeus* from Paengaroa.

Table S6 Individual transcripts comprising each enriched gene ontology category as a result of cold-shock treatment in two species of *Micrarchus* stick insects.

Table S7 Individual identifier and collection location for each *Micrarchus* sample.

Fig. S1 Top twelve most frequently hit species for BLASTx annotation (*E*-value < 1-10) against the NCBI non-redundant protein database using the single top-match for each contig from the three separate de novo assembled transcriptomes.

Fig. S2 Relative distribution of level two gene ontology (GO) annotations between the different *Micrarchus* transcriptome assemblies.

Fig. S3 Expanded *Micrarchus* 28S Bayesian phylogeny.

Fig. S4 Expanded *Micrarchus* COI/COII Bayesian phylogeny.

Fig. S5 Validation of RNA-Seq expression data with qPCR technical and biological replicates for three populations representing two species of *Micrarchus* stick insect.

Appendix S1 Significant expression results from *Micrarchus* nov. sp. 2 (Mt. Arthur).