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Luke T Dunning
University of London - Imperial College of Science, Technology and Medicine, L.Dunning@imperial.ac.uk

Alice B Dennis
Landcare Research, Auckland, New Zealand

Brent J Sinclair
Western University, bsincla7@uwo.ca

Richard D Newcomb
University of Auckland

Thomas R Buckley
University of Auckland

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Molecular Ecology

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

Running Title (max 45 characters inc. spaces):

Cold-induced changes to transcription

Luke T. Dunning[1,2,3,4], Alice B. Dennis[1,3,5,], Brent J. Sinclair[6], Richard D. Newcomb[2,3,7], Thomas R. Buckley[1,2,3]

[1]Landcare Research, Private Bag 92170, Auckland, New Zealand

[2]School of Biological Sciences, University of Auckland, Auckland, New Zealand

[3]Allan Wilson Centre for Molecular Ecology and Evolution, New Zealand

[4] Present address: Imperial College London, Silwood Park, Berkshire, UK

[5] Present address: EAWAG, Swiss Federal Institute of Aquatic Science and Technology; 8600 Dübendorf, Switzerland

[6]Department of Biology, The University of Western Ontario, London, ON, Canada N6G 1L3

[7]The New Zealand Institute for Plant & Food Research Limited, Auckland, New Zealand

Corresponding Author: Luke T. Dunning (L.Dunning@imperial.ac.uk). Imperial College London, Silwood Park Campus, Buckhurst Road, Ascot, Berks, SL5 7PY, UK

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Abstract (<250 words)

In widespread and genetically-structured populations, temperature variation may promote local adaptation and lead to among-population differentiation of thermal biology. The New Zealand stick insect genus *Micrarchus* contains four species (two of which are geographically widespread) which inhabit different thermal environments. RNA-Seq and qPCR were used to investigate the transcriptional responses to cold-shock (-5°C for 1 h then 21°C for 1 h) among lowland and alpine species to identify cold-responsive transcripts and determine geographic variation in gene expression. We also used DNA sequences and transcriptome-wide SNPs to determine phylogeographic structure and the potential for locally-adapted gene expression. RNA-Seq identified 2,160 unigenes differentially expressed across two alpine populations (both *M.* nov. sp. 2) and one lowland population (*M. hystriculeus*), with a majority (68% ± 20%) being population- and species-specific. Responses to cold-shock shared among genetically-divergent *M.* nov. sp. 2 populations that differ from *M. hystriculeus* included the enrichment of cuticular structure-associated transcripts, suggesting that cuticle modification may have accompanied colonisation of the low-temperature alpine environment by *M.* nov. sp. 2. We further show with qPCR that cold-induced changes to transcription in *M.* nov. sp. 2 was significantly correlated across years, suggesting that the differences we observed are a consequence of divergence in genetic background. One alpine population maintains a putatively pure nuclear genome despite complete *M. hystriculeus* mtDNA replacement, possibly indicating local adaptation in the nuclear genome. These results show that, across a relatively small spatial scale, reduced gene flow and possible local adaptation are associated with location-specific transcriptional responses to temperature.

Introduction

The thermal environment, especially the frequency, intensity and duration of low-temperature exposure, can vary considerably across a species' geographic range (Kingsolver 1989; Chown & Terblanche 2006). In genetically-structured populations with low rates of gene flow, variation in thermal stress may promote local adaptation and differentiation of their thermal biology (Ghalambor *et al.* 2007; Sinclair 2012). This differentiation is likely to be manifested in modifications to protein-coding sequences (Dahlhoff & Rank 2000) and gene expression profiles (Hoffmann & Willi 2008) at temperature-related loci. The global transcriptional responses to thermal stress have been widely studied in *Drosophila*, with fewer studies in other insect taxa (Oleksiak *et al.* 2002; Hoffmann *et al.* 2003). Microarray and RNA-Seq studies in intertidal copepods (*Tigriopus californicus*) (Schoville *et al.* 2012) and reef-building coral larvae (*Montastraea faveolata*) (Polato *et al.* 2010) reveal strikingly divergent transcription responses to thermal stress among populations. In *M. faveolata,* this has been attributed to local adaptation occurring in spite of high gene flow among populations (Polato *et al.* 2010). Species comprised of multiple genetically-isolated populations may develop divergent locally-adapted transcriptional profiles, and are therefore likely to respond to climate change differently than more genetically-homogeneous species (Pelini *et al.* 2009; Sinclair 2012). High-altitude endemic species from temperate mountain ranges are at a disproportionally high risk from climate change because treeline expansion is predicted to dramatically reduce suitable habitat (Dirnböck *et al.* 2011). One proposed solution to this threat is translocations between mountain tops (Weeks *et al.* 2011). However, managed translocations may result in reduced fitness if geographic genetic variation is locally-adapted (Weeks *et al.* 2011), thus, determining the occurrence and prevalence of local adaptation is essential for managing populations in the face of climate change.

Insects have successfully colonised the complete range of terrestrial environments, including polar and alpine habitats where they experience sub-zero conditions (Sømme 1995). Sub-zero 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 hemolymph (MacMillan *et al.* 2012). Microarray studies on laboratory *Drosophila* populations have identified cold-responsive loci with functions relating to 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 *Drosophila* cold tolerance genes do not overlap with those identified from the limited number of studies using other insects (Colinet *et al.* 2007; Purać *et al.* 2008; Clark *et al.* 2009; Teets *et al.* 2012; Dunning *et al.* 2013a). For example, *hsp83* (a homolog of mammalian *hsp90*) is up-regulated by *D. melanogaster* adults 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 *Drosophila virilis* and *D. montana* share only two consistently differentially-expressed genes in response to cold acclimation (14 days at 19°C followed by six days at 5°C) and rapid cold hardening (20 days at 19°C followed by 1 h at 0°C) (Vesala *et al.* 2012). Using a phylogenetic framework to investigate inter- and intra-specific 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 geographic range of a species and whether there is variation in this response which could be driven by divergent selection.

We hypothesise that species with poor dispersal ability are likely to have strong phylogeographic structure, and locally-adapted responses to environmental stress may evolve if this structure overlies significant variation in environmental conditions. Apterous (wingless) New Zealand stick insects from the genus *Micrarchus* Carl (Carl 1913) are therefore a great model to investigate if poor dispersal, resulting in strong phylogeographic structure, does result in significant inter- and intraspecific variation in the gene expression response to low temperature. *Micrarchus* is a genus of New Zealand stick insects comprised of four endemic species that all overwinter as nymphs and/or adults. All four species experience sub-zero 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 species (Salmon 1991; Dennis *et al.* 2013). *Micrarchus* nov. sp. 2 is exclusively found at high elevations (650 to 1400 m a.s.l) in contrast to the ecological generalist *M.* nov. sp. 1 (0 to 1100 m a.s.l), and lowland *M. hystriculeus* and *M.* nov. sp. 3 (0 to 283 m a.s.l) (Salmon 1991; Dennis *et al.* 2013). Montane populations of *M.* nov. sp. 2 are effectively isolated on alpine 'sky islands' (Heald 1951). As *Micrarchus* species are apterous there is little 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.* 2013). 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) that encode: 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 likely reflect fixed molecular mechanisms that may be associated with the colonisation of the colder alpine environment, especially if they are not shared with a closely related lowland species. Additionally, Geographically-isolated alpine populations of *M.* nov. sp. 2 offer an opportunity to investigate whether reduced gene flow between populations results in the differentiation of cold-induced transcription as local adaptation will not be swamped by admixture with unadapted genotypes. There is presently little understanding of how thermal adaptation varies over the geographic range of a species, whether populations have locally adapted thermal biologies, and how these differences may be divergently acted upon by climate change-imposed selection

Materials and Methods

Sample collection

Micrarchus specimens were collected across their known distributions between 2004 and 2012 (Fig. 1; Full location details Table S1). The nominate *M. hystriculeus* (Westwood 1859) has the broadest geographical distribution (Fig. 1) and is the only representative 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.* 2013). *Micrarchus* nov. sp. 2 (NZAC03009458) is exclusively found at high elevations (600 to 1409 m above sea level) in the mountains of the northwestern South Island (Salmon 1991; Dennis *et al.* 2013; 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.* 2013). Insects were preserved in ethanol or transported live to Landcare Research, Auckland.

Micrarchus *phylogeny reconstruction*

To assess the degree of phylogeographic structure among and within *Micrarchus* species, and therefore the potential for local adaptation, we collected nuclear and mitochondrial DNA sequence data. DNA was extracted from 5 – 10 mg of leg muscle tissue using the Corbett X-tractor Gene robot (Corbett Robotics, Brisbane, Australia) and the QIAxtractor DX Reagents kit (Qiagen, Hilden, Germany, cat. no. 950107) following the manufacturer's instructions for 148 individuals. Mitochondrial 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 sequence 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.* 2010; Dunning *et al.* 2013b). Sequences were edited and assembled into alignments for each gene using Geneious v.5.6.4 (Drummond *et al.* 2012). Nucleotide substitution models for phylogenetic analysis were selected based on the corrected Akaike Information Criterion (Sugiura 1978; Hurvich & Tsai 1989) implemented in jModelTest v. 0.1.1 (Guindon & Gascuel 2003; Posada 2008). Bayesian phylogenies were constructed using Markov-chain Monte Carlo (MCMC) sampling in MrBayes v.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 v. 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 alpine *M.* nov. sp. 2 was collected from two sites: (i) Sewell Peak (SP), in the Paparoa Range, on 11/02/2011 (Site 1; Fig. 1 & Table S1) and; (ii) Mt. Arthur (MA), Arthur

Range, on 13/02/2011 (Site 9; Fig. 1 & Table S1). These two allopatric populations are separated by 176 km, much of which is comprised of low elevation forest, in which this species is absent. The lowland *M. hystriculeus* was collected from Paengaroa Scenic Reserve (PA), Taihape, on 25/03/2011 (Site 38; Fig. 1 & Table S1). Prior to experimentation insects were maintained under constant conditions for a minimum of 16 days acclimatisation 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 (For *M.* nov. sp. 2 (SP) and *M. hystriculeus* (PA) n = 3 vs. 3; *M.* nov. sp. 2 (MA) n = 6 vs. 6). Experiments were conducted on 17/03/2011 for *M.* nov. sp. 2 (SP and MA), and on 11/04/2011 for *M. hystriculeus* (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 cold-shocked experimental individuals were incubated at 21°C for one hour prior to cooling at approximately 1°C min^{-1} until the incubator reached -5°C, where it was held for one hour. The animals were then warmed at approximately 1°C min^{-1} and held at 21°C for a one hour recovery, at which point insects were snap frozen and all samples stored at -80°C prior to RNA extraction. 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, cat. No. 610.06) from the head, antennae and prothorax of the 24 experimental samples using previously published methods (Dunning *et al.* 2013a). cDNA libraries for HTS were prepared using the Illumina TruSeq RNA Sample Preparation Kit (Illumina San Diego, CA, USA, cat. no. 15013136) 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 high throughput sequencing (HTS) to the University of Utah's Microarray and Genomic Analysis Shared Resource where they were qPCR quantified prior to sequencing on three lanes of a 50 cycle single-end Illumina HiSeq 2000 run.

Pre-processing, aligning and annotating RNA-Seq data

Reads with ambiguous bases were removed using ShortRead v. 1.16.3 (Morgan *et al.* 2009) implemented in R v. 2.15.0 (R Development Core Team 2012). Illumina adapter sequences and low quality bases (Phred quality < 30) were trimmed using Cutadapt v. 1.1 (Martin 2011). Poly A/T tails longer than 10 bp from either end of the reads, and any read shorter than 40 bp was removed using PRINSEQ lite v. 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* Butterfly option used to combine similar splice variants. Ribosomal RNA was removed using all available databases in riboPicker v.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 v. 2.6.2 (Conesa *et al.* 2005). GO annotations were compared and visualised 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 v. 1.2.4 (Li & Dewey 2011). Differential expression analysis followed previously published methods (Dunning *et al.* 2013a). In brief, DESeq v. 1.12.0 (Anders & Huber 2010), edgeR v. 3.2.4 (Robinson *et al.* 2010) and baySeq v. 1.14.1 (Hardcastle & Kelly 2010) packages implemented in R v. 3.0.1 (R Development Core Team 2012) were used to determine which genes were differentially expressed using recommended significance cut-offs to control for Type I errors. 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 (3 out of 6 in *M. hystriculeus* (PA) and *M.* nov. sp. 2 (SP); 6 out of 12 in *M.* nov. sp. 2 (MA)). Gene Ontology (GO) 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 false discovery rate (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, with a range of GO terms and direction of regulation (Table S2), were selected for qPCR using both technical and biological replicates. Two non-differentially expressed reference genes across all three comparisons in the RNA-Seq data were selected to normalise the relative qPCR expression between genes (*pyruvate kinase* and *ATP synthase subunit beta*; *P*-value > 0.85; counts > 1000). Technical replicates used cDNA synthesised from the same individuals as RNA extractions used for the RNA-Seq data. Biological replicates consisted of cDNA synthesised from different individuals from the same

population, with *M.* nov. sp. 2 individuals collected and treated on different dates to the technical replicates; *M.* nov. sp. 2 (SP) collected 27/01/2012 and treated 28/02/2012; *M.* nov. sp. 2 (MA) collected 23/01/2012 and treated 02/03/2012. In addition, three further populations were used for qPCR. These were Alpine *M.* nov. sp. 2 collected from: (i) Mt. Owen (MO), Nelson Range on 25/01/2012 and treated on 01/03/2012 (Site 5; Fig. 1 & Table S1) and; (ii) Harwood's Hole (HH), Takaka Hill on 24/01/2012 and treated on 29/02/2012 (Site 11; Fig. 1 & Table S1). A sample of lowland *M. hystriculeus* was collected from Kowhai (KO), Wairau Valley on 19/01/2012 treated on 25/04/2012 (Site 14; Fig. 1 & Table S1). For biological and population replicates, experimental treatments and RNA extractions followed previously described methods for RNA-Seq samples. Contaminating DNA was removed from RNA extractions using TURBO DNase (Invitrogen, Carlsbad, CA, cat. no. AM2238) prior to cDNA synthesis. Primer design (primer sequences Table S3), cDNA library preparation and qPCR followed previously published methods (Dunning *et al.* 2013a). Fold changes were generated by dividing the mean relative amount for each treatment group by the mean relative amount for the control. Linear regression with Pearson correlation coefficients were 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 similarities calculated. To generate SNP data to compare between the three populations,

a new Trinity assembly was 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 v. 0.1.18 (Li *et al.* 2009). The data set was trimmed to only retain bialleic, unlinked and high quality (Q > 30) SNPs with no missing data using VCFtools v. 0.1.10 (Danecek *et al.* 2011). Population structure was assessed by way of a principle component analysis (PCA) performed in R (R Development Core Team 2012) and ancestry inference using ADMIXTURE v.1.22 (Alexander *et al.* 2009) with K = 2 to K = 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). An average of 9.8 million reads (range: 6.6-13.2 million reads) were generated for each library (Table S4), with 99% of the data remaining after trimming. Cleaned reads were *de novo* assembled by species and population: *M.* nov. sp. 2 (SP), *M.* nov. sp. 2 (MA) and *M. hystriculeus* (PA); producing 42,425 (41,771 unigenes), 58,743 (57,576 unigenes) and 37,814 (37,334 unigenes) contigs, respectively (Table S4). 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). The distribution of high level GO terms from the SwissProt BLASTx matches was extremely similar among the three assemblies (Fig. S2).

Phylogeography and population genetics

A total of 210 individuals were sequenced, resulting in alignments of 530 bp for 28S and 1515 bp of COI/COII. All sequences have been submitted to NCBI Genbank. 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). 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).

For example, the *M.* nov. sp. 2 haplotype clade containing SP, Denniston 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* individuals. *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 *M.* nov. sp. 2 (MA) mtDNA protein coding sequences in the RNA-Seq data have a greater pairwise nucleotide identity to those from *M. hystriculeus* (PA) (average pairwise identity = 95.2%) than the conspecific *M.* nov. sp. 2 (SP) (average pairwise identity = 92.7%), apart from *NADH dehydrogenase subunit 4L* (*ND4L*). The *ND4L* gene is 285 bp in *M.* nov. sp. 2 (MA) and is 98.1% identical to *M. hystriculeus* (PA) (11 observed substitutions) and 98.6% identical to *M.* nov. sp. 2 (SP) (ten observed substitutions). The degree of admixture in the nuclear genome between *M. hystriculeus* and *M.* nov. sp. 2 was assessed using 45,785 biallelic unlinked transcriptome wide SNPs extracted from all 24 individuals from three populations: 6 x *M. hystriculeus* (PA) (Pure *M. hystriculeus* mtDNA); 6 x *M.* nov. sp. 2 SP (pure *M.* nov. sp. 2 mtDNA) and 12 x *M.* nov. sp. 2 (MA) (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 in the data 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 lower fit to the data (higher cross-validation error). These data demonstrate a high degree of differentiation in the nuclear genomes of the species of *Micrarchus*, however, there is strong evidence of gene flow at mitochondrial DNA.

Differential expression analysis

Differentially-expressed transcripts as a result of cold-shock treatment were identified in two alpine populations of *M.* nov. sp. 2 (MA and SP) and one lowland population of *M. hystriculeus* (PA). 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 reads from each individual having at least one valid alignment. Differential expression analyses identified 1774, 252 and 134 differentially expressed cold-responsive unigenes in *M.* nov. sp. 2 (SP), *M.* nov. sp. 2 (MA) and *M. hystriculeus* (PA) 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 (*M.* nov. sp. 2 (MA) 52%; *M.* nov. sp. 2 (SP) 90%; *M. hystriculeus* (PA) 62%; Mean 68% ± 20%), sharing no BLASTx match (*E*-value threshold = $< 1e^{-10}$) with differentially-expressed genes 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). 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 to the non-differentially expressed unigenes was assessed using a Fisher's exact test (FDR <0.05). In *M.* nov. sp. 2 (SP) 32 molecular functions were significantly enriched in the differentially expressed unigenes (Table 2). In *M.* nov. sp. 2 (MA) 3 molecular functions were significantly enriched in the differentially expressed unigenes (Table 2). In *M. hystriculeus* (PA) a single molecular function was significantly enriched in the differentially expressed unigenes (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 *M.* nov. sp. 2 (SP) and *M.* nov. sp. 2 (MA).

qPCR verification

To validate the RNA-Seq results and to further explore geographical patterns of differential expression, expression was also measured in a subset of six genes (Table S2) by qPCR for 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 replicates (*P*-value < 0.001; Fig. 5; Fig. S5). There was also a significant positive correlation between the RNA-Seq and qPCR results for the biological replicates (*P*-value < 0.009; Fig. 5, Fig. S5). The expression of the qPCR candidates was also assessed in alpine *M.* nov. sp. 2 (MO) and *M.* nov. sp. 2 (HH), and lowland *M. hystriculeus* (KO) (Fig. 5) to obtain further information on intraspecific patterns of gene expression. There was a high level of intra- and inter-specific variation in the magnitude of expression response to the cold-shock treatment. For example, in *Endocuticle structural glycoprotein db-2* (*Cud2*) the direction of regulation in the additional alpine *M.* nov. sp. 2 populations were consistent with *M.* nov. sp. 2 (MA), but in only *M.* nov. sp.

2 (HH) was the gene significantly down-regulated as a result of cold-shock. *Sarcosine dehydrogenase* (*Sardh*) is significantly up-regulated in *M.* nov. sp. 2 (HH), but not in *M.* nov. sp. 2 (MO); even though the overall fold-change is higher in the latter. *Cathepsin L* is significantly up-regulated in three of the four alpine populations (*M.* nov. sp. 2 (MA), *M.* nov. sp. 2 (SP) and *M.* nov. sp. 2 (MO)). *Unannotated contig8923* (*Comp8923*) is up-regulated as a result of cold-shock in both populations of the alpine species.

Discussion

All *Micrarchus* experience sub-zero 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 experienced by its congeners (Salmon 1991; Dennis *et al.* 2013). Thus, we expect *M.* Nov. sp. 2 to have a more cold-hardy genotype. In the context of the likely restricted gene flow imposed by the sky island distribution of this species, we also expect local adaptation of the gene expression response to low temperature. In this study we show that, across a relatively small spatial scale, reduced gene flow and possible local adaptation are associated with location-specific transcriptional responses to low temperature exposure.

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, a prerequisite for local adaptation. All species are monophyletic at 28S, and transcriptome wide SNPs 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 mtDNA introgression in *Micrarchus*. Reduced nuclear introgression may indicate local adaptation in *M.* nov. sp. 2, with selection against maladapted *M. hystriculeus* nuclear genes in *M.* nov. sp. 2 populations (Nosil *et al.* 2005).

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). However, extensive variation in the transcriptional response to cold-shock mirrors the genetic diversity witnessed in *Micrarchus*, with 68% (± 20%) of 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 alpine *M.* nov. sp. 2, but not in lowland *M. hystriculeus*. In the two genetically distinct and geographically isolated populations of *M.* nov. sp. 2 used for RNA-Seq, structural cuticle unigenes are predominately differentially regulated in opposite directions. Geographically-isolated populations of marine copepods show similar disparate responses to thermal stress in genes associated with cuticle structure (Schoville *et al.* 2012). Structural reorganisation of the cuticle in response to cold-shock in *M.* nov. sp. 2 may be an adaptation that has facilitated the colonisation of the alpine environment and the development of a more cold-hardy phenotype.

At the individual cuticle unigene level, both *M.* nov. sp. 2 (MA) and *M.* nov. sp. 2 (SP) up-regulate two *cathepsin L* (*Catl*) orthologous 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

414 cyroprotective dehydration (Clark *et al.* 2009). In addition, cuticular genes are significantly
415 differentially expressed in the qPCR experiments in *M.* nov. sp. 2 (MO) (*Catl*) and *M.* nov. sp. 2
416 (HH) (*Cud2*). We previously identified a cold-responsive cuticular protein upregulated by *M.*
417 nov. sp. 2 in response to a mild cold-shock (1 h 0°C with 1 h recovery at 20°C) (Dunning *et al.*
418 2013a). However, this gene (*cuticular protein analogous to peritrophins 3-d2*) was not
419 differentially expressed in the current study, illustrating variation in the transcriptional response
420 to mild and severe cold-shock consistent with other physiological differences between different
421 intensities of cold exposure (reviewed by Sinclair & Roberts 2005). Cold-responsive cuticular
422 genes and proteins have been identified in many other insect species including flies (Qin *et al.*
423 2005), wasps (Colinet *et al.* 2007), beetles (Carrasco *et al.* 2011), and locusts (Wang *et al.* 2012);
424 indicating that changes in this tissue likely plays an important role in the adaptation to low-
425 temperature, however, the physiological role of the cuticle in cold tolerance has not yet been
426 explored.

427 *The evolution of gene expression in a phylogenetic context*

428 The differential expression of cuticular genes appears to be an evolutionarily-conserved
429 response to low-temperature exposure across all populations of cold-hardy alpine *M.* nov. sp. 2.
430 However, a majority of other differentially expressed unigenes are population-specific. These *M.*
431 nov. sp. 2 population-specific responses are consistent among biological replicates collected
432 from the wild in different years, even though these individuals would likely have experienced
433 differing environmental regimes during development. This indicates that the genetic structuring
434 of populations across the mountain ranges as a result of reduced gene flow likely promotes the
435 differentiation of their transcriptional profiles. Furthermore the integrity of the nuclear genome
436 in *M.* nov. sp. 2 is maintained despite a complex pattern of unidirectional mitochondrial

replacement from the lowland *M. hystriculeus*. This provides some evidence for local adaptation in *M.* nov. sp. 2, with selection against maladapted introgresing *M. hystriculeus* nuclear genes. Small population sizes and extremely low migration rates increase the chance of fixation of locally adapted traits that have even relatively small positive selective coefficients (Lenormand 2002; Kawecki & Ebert 2004). This raises the possibility that some of the fixed expression differences between *M.* nov. sp. 2 populations are a result of local adaptation.

While the 'core' transcriptional response of cuticular gene expression has been maintained, the variation in low-temperature response may mean that populations of *M.* nov. sp. 2 will respond differently to future altered climate regimes. This is contrary to genetically-homogenous species where high dispersal between populations will prevent the maintenance of local alleles and lead to a more uniform response (Case & Taper 2000). The evolution of divergent transcriptional responses to low-temperature exposure in isolated populations of *M.* nov. sp. 2 has two important implications. First, this divergence has evolved in response to presumably similar montane environmental stressors from a common genetic background, which implies that these two populations have solved the physiological challenges of montane habitats in different ways. Thus, we show that there may be significant evolutionary divergence in the responses to abiotic stressors even when the environmental stressors are ostensibly similar. Second, local adaptation in response to environmental stressors presents challenges for managing populations in the face of climate change, because locally-adapted genotypes may be unsuitable for translocation to new locations, and may even perform poorly as their current habitat changes. While such local adaptation has been identified in organismal-level studies (e.g. Pelini *et al.* 2009), our work suggests that it may be possible to identify functional local adaptation by screening gene expression responses to simple abiotic stressors.

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637

638 **Tables**

639 **Table 1:** Differentially expressed cold-shock responsive transcripts in *Micrarchus* stick insects
640 identified using RNA-Seq. Cold-shock treated individuals (21°C for 1 h; -1°C min^{-1} for 26 min; -
641 5°C for 1 h; +1°C min^{-1} for 26 min; 21°C for 1 h) were compared to controls (21°C for 3 h) using
642 three exact test approaches (edgeR with common dispersion, edgeR with tagwise dispersion and
643 DESeq) and a Bayesian methods (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	1,077	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**	**1,350**	**78**	**174**	**94**	**40**

644 Significance cut-off for: edgeR = FDR < 0.05 (*P*-value < 0.003); DESeq = FDR <0.10 (*P*-value < 0.002); BaySeq FDR <0.10
645 (likelihood > 0.75). * = number of unigenes used for differential expression analysis after removing unigenes with less than one
646 count per million in at least three samples for Sewell Peak/Paengaroa and 6 samples for Mt. Arthur, † = using common
647 dispersion, ‡ = using tagwise dispersion. Total = unique unigenes called by all analysis methods.

648

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 at least one analysis (Table 1) in the cold-shock treatment (21°C for 1 h; -1°C min^{-1} for 26 min; -5°C for 1 h; +1°C min^{-1} for 26 min; 21°C for 1 h) and control (21°C for 3 h) groups 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*-Value	#DR	#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 signaling	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	Hemoglobin 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; **#CON** = Number of differentially regulated unigenes with increased expression in control group; **#GO** = total number in category; * = acting on paired donors, with incorporation or reduction of molecular oxygen, another compound as one donor, and incorporation of one atom of oxygen.

Figure Captions

Fig. 1: Geographic distribution of the four *Micrarchus* species within New Zealand and their evolutionary relationship based on 28S ribosomal RNA sequences. The 28S Bayesian phylogeny is collapsed into the four species nodes, each with number of samples (*n*), number of different haplotypes (*h*) and percentage pairwise identity between haplotypes (*i*) shown. Upper support values are posterior probabilities estimated with MrBayes; lower support values are likelihood bootstraps (100) estimated with GARLI. 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. Sites used for RNA-Seq and qPCR are named.

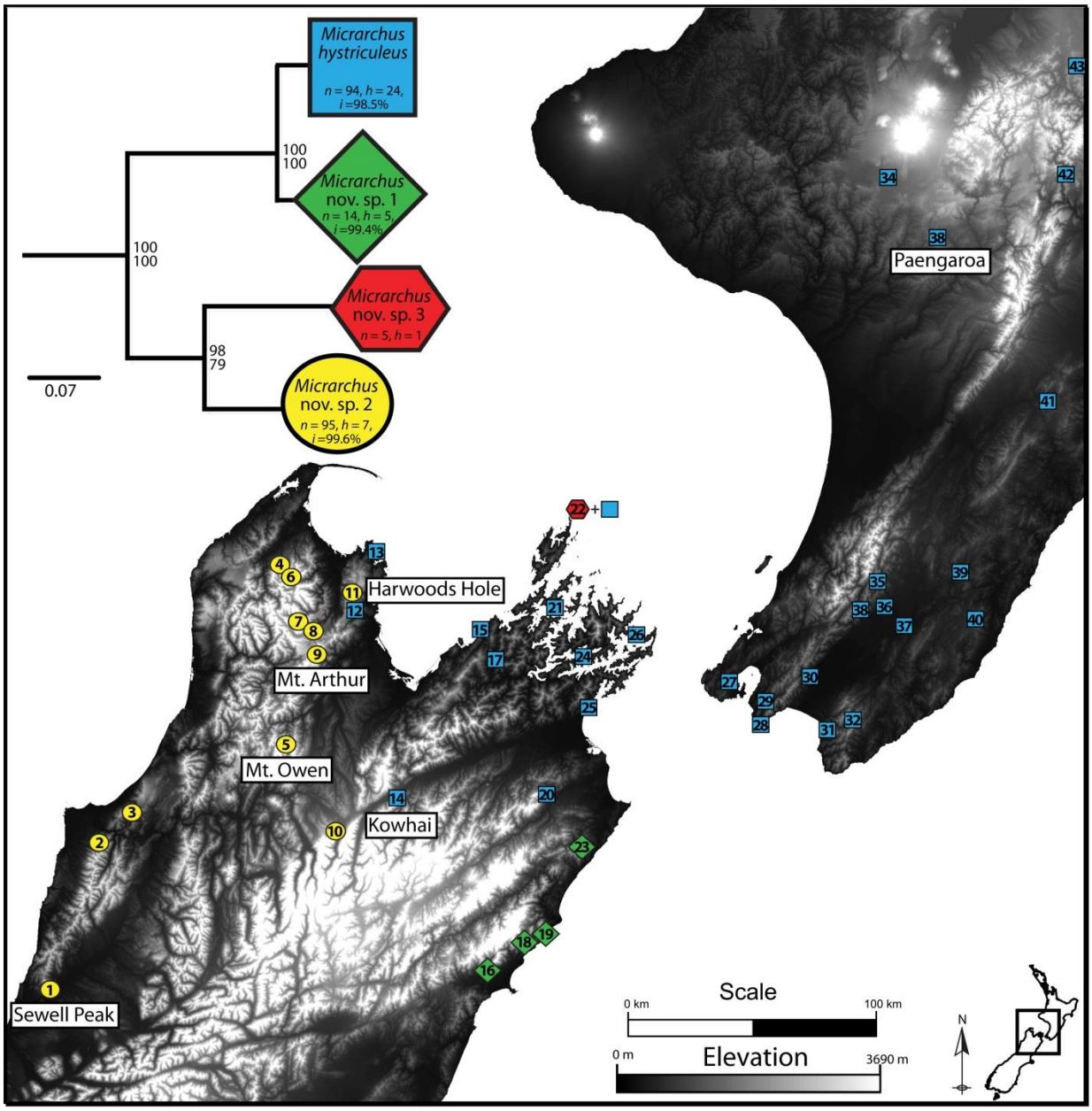
Fig 2: Bayesian phylogeny constructed using mitochondrial cytochrome oxidase subunit I (COI) and II (COII) DNA sequences representing 208 individuals from 43 populations and four *Micrarchus* species. Nodes collapsed to population, with those that are non-monophyletic denoted with an asterisk. Support values are posterior probabilities estimated in MrBayes. Scale bar represents the number of substitutions per site. Sites used for RNA-Seq and qPCR are named. The full detail of each of the locations, samples and the expanded phylogeny is provided in Table S1 and Fig. S4.

Fig 3: a) Principle components analysis and b) Inferred ancestry (Q-Plot) based on 45,785 biallelic unlinked SNPs from 24 individuals representing three populations and 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).

Fig. 4: Intra- and inter-specific 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*-value $<1^{-10}$) sequence match with a differentially expressed unigenes in the other populations. Cold-shock treatment = 21°C for 1 h; -1°C min-1 for 26 min; -5°C for 1 h; +1°C min-1 for 26 min; 21°C for 1 h. Control treatment = 21°C for 3 h. If a unigene had more than one BLASTx match only the match with the highest *E*-value was used.

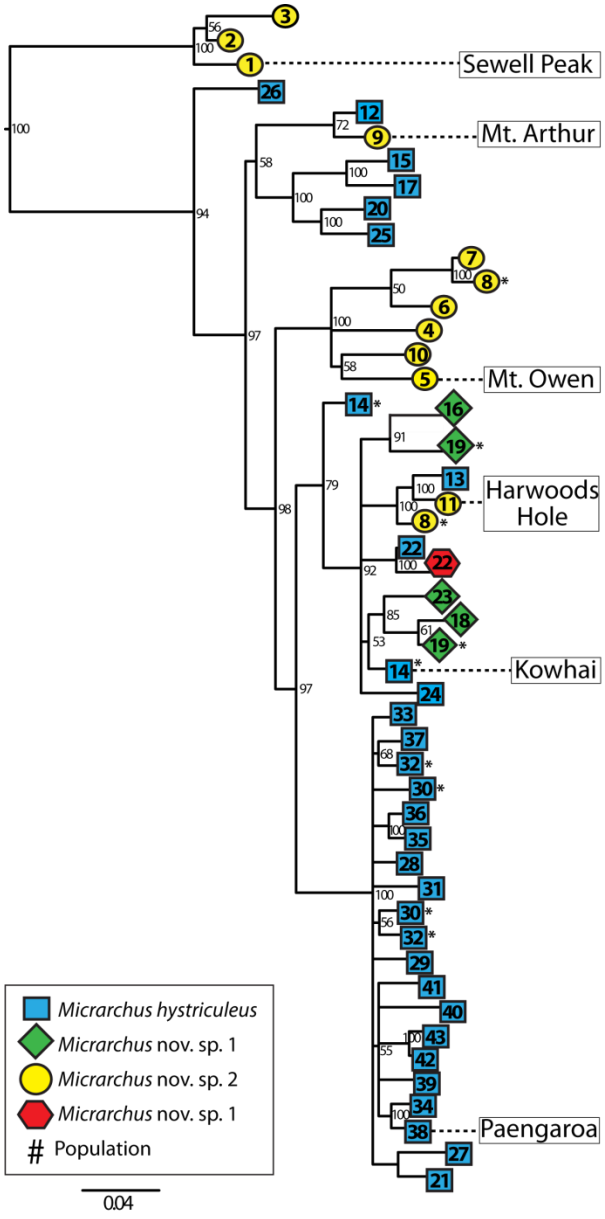
Fig. 5: Heat map representing log transformed fold-changes in gene expression between control (21°C for 3 h) and cold shocked (21°C for 1 h; -1°C min^{-1} for 26 min; -5°C for 1 h; +1°C min^{-1} for 26 min; 21°C for 1 h) *Micrarchus* stick insects using RNA-Seq and qPCR. * = Significantly differentially expressed (qPCR samples t-test *P*-value < 0.05; RNA-Seq samples FDR < 0.05 edgeR or < 0.10 baySeq/DESeq. qPCR mRNA abundances normalised using two reference genes (*pyruvate kinase* and *ATP synthase subunit beta*). Cartoons represent COI/COII and 28S *Micrarchus* phylogenies, dotted line represents reticulated branch. Negative fold-changes represent increased expression in treatment group (21°C for 1 h; -1°C min-1 for 26 min; -5°C for 1 h; +1°C min-1 for 26 min; 21°C for 1 h; positive fold-changes represent increased expression in control group (21°C for 3 h). Inf = Infinite fold-change as a result of no recorded expression in one sample.

703 **Fig. 1**


Micrarchus hystriculeus
n = 94, h = 24, i =98.5%
Micrarchus nov. sp. 1
n = 14, h = 5, i =99.4%
Micrarchus nov. sp. 3
n = 5, h = 1
Micrarchus nov. sp. 2
n = 95, h = 7, i =99.6%
100
100
100
100
98
79
0.07
Harwoods Hole
Mt. Arthur
Mt. Owen
Kowhai
Sewell Peak
Paengaroa
Scale
0 km
100 km
0 m
Elevation
3690 m
N

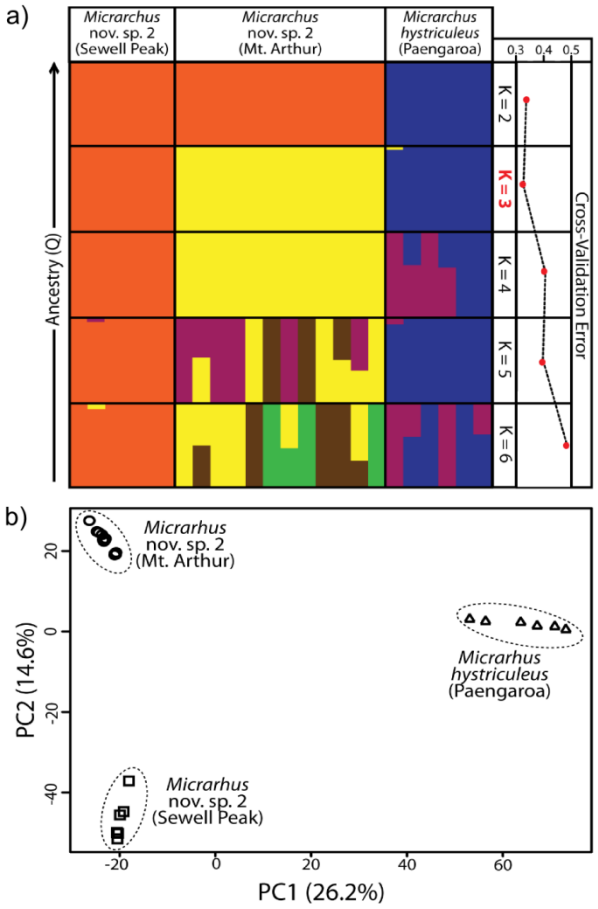
704

705 **Fig. 2**

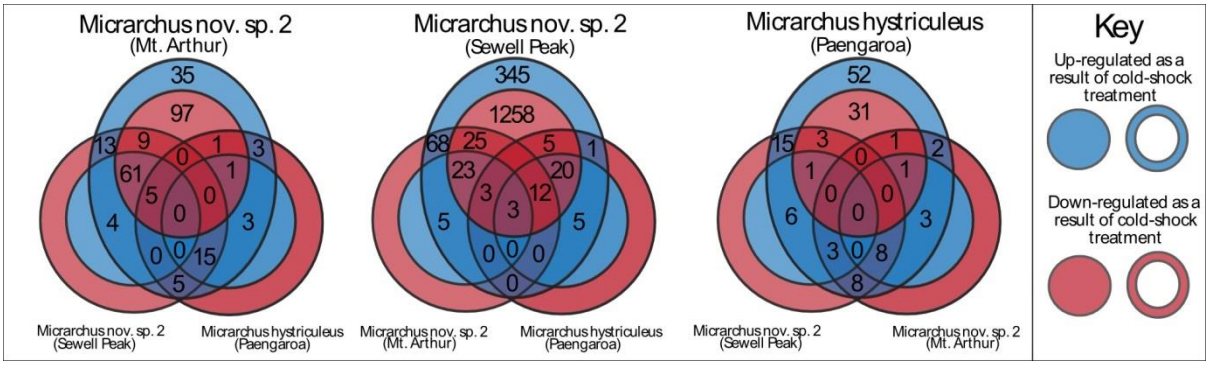

Sewell Peak
Mt. Arthur
Mt. Owen
Harwoods Hole
Kowhai
Paengaroa
Micrarchus hystriculeus
Micrarchus nov. sp. 1
Micrarchus nov. sp. 2
Micrarchus nov. sp. 1
Population
0.04

706

Fig. 3


a)
Micrarchus nov. sp. 2 (Sewell Peak)
Micrarchus nov. sp. 2 (Mt. Arthur)
Micrarchus hystriculeus (Paengaroa)
Ancestry (Q)
K = 2
K = 3
K = 4
K = 5
K = 6
0.3
0.4
0.5
Cross-Validation Error
b)
Micrarhus nov. sp. 2 (Mt. Arthur)
Micrarhus hystriculeus (Paengaroa)
Micrarhus nov. sp. 2 (Sewell Peak)
PC2 (14.6%)
PC1 (26.2%)
20
0
-20
-40
-20
0
20
40
60

710 **Fig. 4**

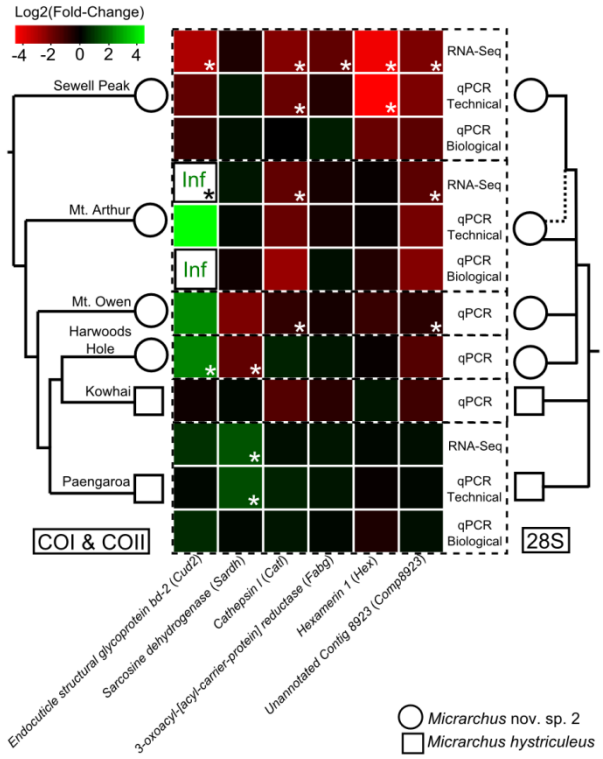


711

712

713

714 **Fig. 5**



715