View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Scholarship@Western

Western University
Scholarship@Western

Biology Publications

Biology Department

6-1-2014

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

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

Follow this and additional works at: https://ir.lib.uwo.ca/biologypub

Part of the Biology Commons, Ecology and Evolutionary Biology Commons, and the Entomology Commons

Citation of this paper:
Dunning, L. T., Dennis, A. B., Sinclair, B. J., Newcomb, R. D. and Buckley, T. R. (2014), Divergent transcriptional responses to low temperature among populations of alpine and lowland species of New Zealand stick insects (Micrarchus). Mol Ecol, 23: 2712–2726. doi:10.1111/mec.12767

1 Molecular Ecology

2 Divergent transcriptional responses to low temperature among populations of alpine and 3 lowland species of New Zealand stick insect (*Micrarchus*)

4 *Running Title (max 45 characters inc. spaces):*

5 **Cold-induced changes to transcription**

6

7 Luke T. Dunning[1,2,3,4], Alice B. Dennis[1,3,5,], Brent J. Sinclair[6], Richard D. Newcomb[2,3,7], Thomas 8 R. Buckley[1,2,3]

9 [1]Landcare Research, Private Bag 92170, Auckland, New Zealand

10 [2]School of Biological Sciences, University of Auckland, Auckland, New Zealand

11 [3]Allan Wilson Centre for Molecular Ecology and Evolution, New Zealand

12 [4] Present address: Imperial College London, Silwood Park, Berkshire, UK

13 [5] Present address: EAWAG, Swiss Federal Institute of Aquatic Science and Technology; 8600 Dübendorf, 14 Switzerland

15 [6]Department of Biology, The University of Western Ontario, London, ON, Canada N6G 1L3

16 [7]The New Zealand Institute for Plant & Food Research Limited, Auckland, New Zealand

17 Corresponding Author: Luke T. Dunning (L.Dunning@imperial.ac.uk). Imperial College 18 London, Silwood Park Campus, Buckhurst Road, Ascot, Berks, SL5 7PY, UK

19 **Keywords:** Cold tolerance, Cuticle, introgression, local adaptation, RNA-Seq

20 This is the pre-peer reviewed version of the following article: Dunning, L. T., Dennis, A. B., Sinclair, B. J., Newcomb, R. D. and 21 Buckley, T. R. (2014), Divergent transcriptional responses to low temperature among populations of alpine and lowland species 22 of New Zealand stick insects (*Micrarchus*). Mol Ecol, 23: 2712–2726. doi:10.1111/mec.12767, which has been published in final 23 form at 10.1111/mec.12767. This article may be used for non-commercial purposes in accordance with Wiley Terms and 24 Conditions for Self-Archiving

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.

48 **Introduction**

49 The thermal environment, especially the frequency, intensity and duration of low-
50 temperature exposure, can vary considerably across a species' geographic range (Kingsolver
51 1989; Chown & Terblanche 2006). In genetically-structured populations with low rates of gene
52 flow, variation in thermal stress may promote local adaptation and differentiation of their thermal
53 biology (Ghalambor *et al.* 2007; Sinclair 2012). This differentiation is likely to be manifested in
54 modifications to protein-coding sequences (Dahlhoff & Rank 2000) and gene expression profiles
55 (Hoffmann & Willi 2008) at temperature-related loci. The global transcriptional responses to
56 thermal stress have been widely studied in *Drosophila*, with fewer studies in other insect taxa
57 (Oleksiak *et al.* 2002; Hoffmann *et al.* 2003). Microarray and RNA-Seq studies in intertidal
58 copepods (*Tigriopus californicus*) (Schoville *et al.* 2012) and reef-building coral larvae
59 (*Montastraea faveolata*) (Polato *et al.* 2010) reveal strikingly divergent transcription responses to
60 thermal stress among populations. In *M. faveolata,* this has been attributed to local adaptation
61 occurring in spite of high gene flow among populations (Polato *et al.* 2010). Species comprised
62 of multiple genetically-isolated populations may develop divergent locally-adapted
63 transcriptional profiles, and are therefore likely to respond to climate change differently than
64 more genetically-homogeneous species (Pelini *et al.* 2009; Sinclair 2012). High-altitude endemic
65 species from temperate mountain ranges are at a disproportionally high risk from climate change
66 because treeline expansion is predicted to dramatically reduce suitable habitat (Dirnböck *et al.*
67 2011). One proposed solution to this threat is translocations between mountain tops (Weeks *et al.*
68 2011). However, managed translocations may result in reduced fitness if geographic genetic
69 variation is locally-adapted (Weeks *et al.* 2011), thus, determining the occurrence and prevalence
70 of local adaptation is essential for managing populations in the face of climate change.

71 Insects have successfully colonised the complete range of terrestrial environments,
72 including polar and alpine habitats where they experience sub-zero conditions (Sømme 1995).
73 Sub-zero temperature exposure induces physiological and biochemical stresses including
74 increased membrane rigidity (Overgaard *et al.* 2005), induced apoptosis (Denlinger & Lee 2010),
75 elevated oxidative stress (Lalouette *et al.* 2011) and loss of sodium ions and water from the
76 hemolymph (MacMillan *et al.* 2012). Microarray studies on laboratory *Drosophila* populations
77 have identified cold-responsive loci with functions relating to gene regulation, immune function,
78 metabolism, stress, cuticles, membranes and the cytoskeleton (Qin *et al.* 2005; Laayouni *et al.*
79 2007; Sørensen *et al.* 2007; Zhang *et al.* 2011; Vesala *et al.* 2012). A majority of these candidate
80 *Drosophila* cold tolerance genes do not overlap with those identified from the limited number of
81 studies using other insects (Colinet *et al.* 2007; Purać *et al.* 2008; Clark *et al.* 2009; Teets *et al.*
82 2012; Dunning *et al.* 2013a). For example, *hsp83* (a homolog of mammalian *hsp90*) is up-
83 regulated by *D. melanogaster* adults exposed to 0 °C for 2 h (Qin *et al.* 2005), but not in alpine
84 stick insects (*Micrarchus* nov. sp. 2) exposed to a similar treatment (Dunning *et al.* 2013a; 0°C
85 for 1 h followed by a 1 h recovery period at 20°C). Even within *Drosophila*, patterns of cold-
86 induced gene expression differ among species. For example, the allopatric *Drosophila virilis* and
87 *D. montana* share only two consistently differentially-expressed genes in response to cold
88 acclimation (14 days at 19°C followed by six days at 5°C) and rapid cold hardening (20 days at
89 19°C followed by 1 h at 0°C) (Vesala *et al.* 2012). Using a phylogenetic framework to
90 investigate inter- and intra-specific variation in cold-induced transcription in closely-related
91 species and populations inhabiting different thermal environments will increase our
92 understanding of how cold-tolerance varies across the geographic range of a species and whether
93 there is variation in this response which could be driven by divergent selection.

94 We hypothesise that species with poor dispersal ability are likely to have strong
95 phylogeographic structure, and locally-adapted responses to environmental stress may evolve if
96 this structure overlies significant variation in environmental conditions. Apterous (wingless)
97 New Zealand stick insects from the genus *Micrarchus* Carl (Carl 1913) are therefore a great
98 model to investigate if poor dispersal, resulting in strong phylogeographic structure, does result
99 in significant inter- and intraspecific variation in the gene expression response to low
100 temperature. *Micrarchus* is a genus of New Zealand stick insects comprised of four endemic
101 species that all overwinter as nymphs and/or adults. All four species experience sub-zero
102 temperatures in their respective environments, but the frequency, duration and extremes in
103 temperature encountered by *M.* nov. sp. 2 are greater than those of the other species (Salmon
104 1991; Dennis *et al.* 2013). *Micrarchus* nov. sp. 2 is exclusively found at high elevations (650 to
105 1400 m a.s.l) in contrast to the ecological generalist *M.* nov. sp. 1 (0 to 1100 m a.s.l), and
106 lowland *M. hystriculeus* and *M.* nov. sp. 3 (0 to 283 m a.s.l) (Salmon 1991; Dennis *et al.* 2013).
107 Montane populations of *M.* nov. sp. 2 are effectively isolated on alpine 'sky islands' (Heald
108 1951). As *Micrarchus* species are apterous there is little potential for migration or gene flow
109 among these populations. In July, alpine sites are on average 3.6°C colder and experience ten
110 times more freeze-thaw cycles than nearby lowland sites (Dennis *et al.* 2013). Previously, RNA-
111 Seq has been used to identify three differentially expressed genes in *M.* nov. sp. 2 from one
112 population (Sewell Peak) after brief exposure to a mild cold-shock (0°C) that encode: an
113 oxidoreductase enzyme, a transcriptional regulator and a cuticle protein (Dunning *et al.* 2013a).
114 No information on the molecular response to cold is available for the other three *Micrarchus*
115 species or further populations of *M.* nov. sp. 2.

116 Similarities in the transcriptional response to low-temperature exposure shared among
117 populations of alpine stick insects likely reflect fixed molecular mechanisms that may be
118 associated with the colonisation of the colder alpine environment, especially if they are not
119 shared with a closely related lowland species. Additionally, Geographically-isolated alpine
120 populations of *M.* nov. sp. 2 offer an opportunity to investigate whether reduced gene flow
121 between populations results in the differentiation of cold-induced transcription as local
122 adaptation will not be swamped by admixture with unadapted genotypes. There is presently little
123 understanding of how thermal adaptation varies over the geographic range of a species, whether
124 populations have locally adapted thermal biologies, and how these differences may be
125 divergently acted upon by climate change-imposed selection

126

127 **Materials and Methods**

128 *Sample collection*

129 *Micrarchus* specimens were collected across their known distributions between 2004 and 130 2012 (Fig. 1; Full location details Table S1). The nominate *M. hystriculeus* (Westwood 1859) 131 has the broadest geographical distribution (Fig. 1) and is the only representative on the North 132 Island (Salmon 1991). *Micrarchus* nov. sp. 1 (Voucher specimen NZAC 03000433 from New 133 Zealand Arthropod Collection, Landcare Research, Auckland, New Zealand) is restricted to 134 habitats on the east coast of the South Island (Salmon 1991; Dennis *et al.* 2013). *Micrarchus* 135 nov. sp. 2 (NZAC03009458) is exclusively found at high elevations (600 to 1409 m above sea 136 level) in the mountains of the northwestern South Island (Salmon 1991; Dennis *et al.* 2013; 137 Dunning *et al.* 2013a). *Micrarchus* nov. sp. 3 (NZAC03000053) is only known from near sea 138 level on Stephens Island (150 ha) in the Cook Strait where it is sympatric with *M. hystriculeus* 139 (Buckley *et al.* 2012). Specimens were collected by beating and manually searching host 140 vegetation (Salmon 1991; Dennis *et al.* 2013). Insects were preserved in ethanol or transported 141 live to Landcare Research, Auckland.

142 Micrarchus *phylogeny reconstruction*

143 To assess the degree of phylogeographic structure among and within *Micrarchus* species, 144 and therefore the potential for local adaptation, we collected nuclear and mitochondrial DNA 145 sequence data. DNA was extracted from 5 – 10 mg of leg muscle tissue using the Corbett X-146 tractor Gene robot (Corbett Robotics, Brisbane, Australia) and the QIAxtractor DX Reagents kit 147 (Qiagen, Hilden, Germany, cat. no. 950107) following the manufacturer's instructions for 148 148 individuals. Mitochondrial cytochrome oxidase subunit I (COI), cytochrome oxidase subunit II

149 (COII) and 28S ribosomal RNA genes were sequenced using previously described methods and
150 primers (Buckley *et al.* 2008). Sequence information for these three genes was supplemented
151 with sequence from a further 36 individuals sequenced using cDNA as a template (qPCR
152 samples) and 24 individuals using high-throughput sequencing (RNA-Seq samples). Two
153 *Tectarchus ovobessus* and two *T. salebrosus* specimens were used as outgroups for COI/II and
154 28S respectively. Both of these species are endemic to New Zealand and closely related to
155 *Micrarchus* (Buckley *et al.* 2010; Dunning *et al.* 2013b). Sequences were edited and assembled
156 into alignments for each gene using Geneious v.5.6.4 (Drummond *et al.* 2012). Nucleotide
157 substitution models for phylogenetic analysis were selected based on the corrected Akaike
158 Information Criterion (Sugiura 1978; Hurvich & Tsai 1989) implemented in jModelTest v. 0.1.1
159 (Guindon & Gascuel 2003; Posada 2008). Bayesian phylogenies were constructed using Markov-
160 chain Monte Carlo (MCMC) sampling in MrBayes v.3.2.0 (Huelsenbeck & Ronquist 2001;
161 Ronquist & Huelsenbeck 2003) with 10 million generations sampled every thousand generations.
162 A relative burn-in of 25% was used with the following priors: uniform substitution rates,
163 empirically estimated state frequencies, exponential gamma shape parameter set to mean of five
164 for among-site rate variation and proportion of invariable sites uniformly distributed between
165 zero and one. Non-parametric bootstrap analysis under maximum likelihood (100 pseudo
166 replicates) was performed using Garli v. 2.0 (Zwickl 2006) (substitution model: COI/COII =
167 GTR+I+Γ; 28S = TIM3+Γ).

168 *Cold-shock experiments*

169 Cold-shock experiments were performed exclusively on adult females. For these
170 experiments alpine *M.* nov. sp. 2 was collected from two sites: (i) Sewell Peak (SP), in the
171 Paparoa Range, on 11/02/2011 (Site 1; Fig. 1 & Table S1) and; (ii) Mt. Arthur (MA), Arthur

172 Range, on 13/02/2011 (Site 9; Fig. 1 & Table S1). These two allopatric populations are separated
173 by 176 km, much of which is comprised of low elevation forest, in which this species is absent.
174 The lowland *M. hystriculeus* was collected from Paengaroa Scenic Reserve (PA), Taihape, on
175 25/03/2011 (Site 38; Fig. 1 & Table S1). Prior to experimentation insects were maintained under
176 constant conditions for a minimum of 16 days acclimatisation in a 12:12 light:dark cycle under
177 ambient room temperature and humidity with a constant diet of freshly collected *Metrosideros*
178 *excelsa* leaves. The RNA-Seq experimental design consisted of three independent trials between
179 control and cold-shocked groups (For *M.* nov. sp. 2 (SP) and *M. hystriculeus* (PA) n = 3 vs. 3; *M.*
180 nov. sp. 2 (MA) n = 6 vs. 6). Experiments were conducted on 17/03/2011 for *M.* nov. sp. 2 (SP
181 and MA), and on 11/04/2011 for *M. hystriculeus* (PA). The control groups were maintained at
182 21°C in a Sanyo MIR-154 incubator (Global Science and Technology, Osaka, Japan) prior to
183 being snap-frozen in liquid nitrogen. The cold-shocked experimental individuals were incubated
184 at 21°C for one hour prior to cooling at approximately 1°C min^{-1} until the incubator reached -
185 5°C, where it was held for one hour. The animals were then warmed at approximately 1°C min^{-1}
186 and held at 21°C for a one hour recovery, at which point insects were snap frozen and all
187 samples stored at -80°C prior to RNA extraction. All insects survived the treatments and were
188 moving in a coordinated fashion prior to snap freezing.

189 *cDNA preparation and RNA-Seq*

190 Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA, cat. No. 610.06)
191 from the head, antennae and prothorax of the 24 experimental samples using previously
192 published methods (Dunning *et al.* 2013a). cDNA libraries for HTS were prepared using the
193 Illumina TruSeq RNA Sample Preparation Kit (Illumina San Diego, CA, USA, cat. no.
194 15013136) according to the manufacturer's protocol, with a starting input of 2.65 µg total RNA

195 from each sample. Individual samples had randomly assigned barcodes ligated to the cDNA
196 fragments and were amplified with 13 cycles of PCR. Libraries were validated using the Agilent
197 2100 Bioanalyzer to ensure each had the recommended ~260 bp average fragment size. Libraries
198 were sent for high throughput sequencing (HTS) to the University of Utah's Microarray and
199 Genomic Analysis Shared Resource where they were qPCR quantified prior to sequencing on
200 three lanes of a 50 cycle single-end Illumina HiSeq 2000 run.

201 *Pre-processing, aligning and annotating RNA-Seq data*

202 Reads with ambiguous bases were removed using ShortRead v. 1.16.3 (Morgan *et al.*
203 2009) implemented in R v. 2.15.0 (R Development Core Team 2012). Illumina adapter
204 sequences and low quality bases (Phred quality < 30) were trimmed using Cutadapt v. 1.1
205 (Martin 2011). Poly A/T tails longer than 10 bp from either end of the reads, and any read shorter
206 than 40 bp was removed using PRINSEQ lite v. 0.16 (Schmieder & Edwards 2011). Cleaned
207 reads were grouped by population and *de novo* assembled using Trinity (release 2012-10-05
208 (Grabherr *et al.* 2011)), with default parameters and the *reduce* Butterfly option used to combine
209 similar splice variants. Ribosomal RNA was removed using all available databases in riboPicker
210 v.0.4.3 (Schmieder *et al.* 2012) and BLASTn sequence searching against 28S *Micrarchus*
211 sequences. Assemblies were annotated by BLASTx (*E*-value threshold = $1e^{-10}$) sequence
212 searching against the National Centre for Biotechnology Information (NCBI) non-redundant (*nr*)
213 protein and SwissProt databases. Gene Ontology (GO) annotation was based on the SwissProt
214 Blast matches using Blast2GO v. 2.6.2 (Conesa *et al.* 2005). GO annotations were compared and
215 visualised between assemblies using WEGO (Ye *et al.* 2006).

216 *Differential expression analysis of RNA-Seq data*

217 Individual cleaned sequence reads were mapped back onto the respective population
218 Trinity assemblies using RSEM v. 1.2.4 (Li & Dewey 2011). Differential expression analysis
219 followed previously published methods (Dunning *et al.* 2013a). In brief, DESeq v. 1.12.0
220 (Anders & Huber 2010), edgeR v. 3.2.4 (Robinson *et al.* 2010) and baySeq v. 1.14.1 (Hardcastle
221 & Kelly 2010) packages implemented in R v. 3.0.1 (R Development Core Team 2012) were used
222 to determine which genes were differentially expressed using recommended significance cut-offs
223 to control for Type I errors. To be considered for differential expression analysis 'unigenes'
224 (trinity components containing clusters of 'contigs' representing splice variants of the same
225 locus) had to have at least one count per million in half of the samples analysed (3 out of 6 in *M.*
226 *hystriculeus* (PA) and *M.* nov. sp. 2 (SP); 6 out of 12 in *M.* nov. sp. 2 (MA)). Gene Ontology
227 (GO) annotation enrichment analysis was performed by way of a Fisher's exact test in Blast2GO
228 (Conesa *et al.* 2005) between the GO terms associated with the differentially regulated cold-
229 responsive unigenes, and those associated with the non-differentially-expressed unigenes. The
230 analysis was restricted to biological function with a false discovery rate (FDR) of < 0.10).

231 *qPCR validation*

232 To validate the RNA-Seq results and gain more detailed information on the variation in
233 expression among populations and species a subset of six genes, with a range of GO terms and
234 direction of regulation (Table S2), were selected for qPCR using both technical and biological
235 replicates. Two non-differentially expressed reference genes across all three comparisons in the
236 RNA-Seq data were selected to normalise the relative qPCR expression between genes (*pyruvate*
237 *kinase* and *ATP synthase subunit beta*; P-value > 0.85; counts > 1000). Technical replicates used
238 cDNA synthesised from the same individuals as RNA extractions used for the RNA-Seq data.
239 Biological replicates consisted of cDNA synthesised from different individuals from the same

240 population, with *M.* nov. sp. 2 individuals collected and treated on different dates to the technical
241 replicates; *M.* nov. sp. 2 (SP) collected 27/01/2012 and treated 28/02/2012; *M.* nov. sp. 2 (MA)
242 collected 23/01/2012 and treated 02/03/2012. In addition, three further populations were used for
243 qPCR. These were Alpine *M.* nov. sp. 2 collected from: (i) Mt. Owen (MO), Nelson Range on
244 25/01/2012 and treated on 01/03/2012 (Site 5; Fig. 1 & Table S1) and; (ii) Harwood's Hole
245 (HH), Takaka Hill on 24/01/2012 and treated on 29/02/2012 (Site 11; Fig. 1 & Table S1). A
246 sample of lowland *M. hystriculeus* was collected from Kowhai (KO), Wairau Valley on
247 19/01/2012 treated on 25/04/2012 (Site 14; Fig. 1 & Table S1). For biological and population
248 replicates, experimental treatments and RNA extractions followed previously described methods
249 for RNA-Seq samples. Contaminating DNA was removed from RNA extractions using TURBO
250 DNase (Invitrogen, Carlsbad, CA, cat. no. AM2238) prior to cDNA synthesis. Primer design
251 (primer sequences Table S3), cDNA library preparation and qPCR followed previously
252 published methods (Dunning *et al.* 2013a). Fold changes were generated by dividing the mean
253 relative amount for each treatment group by the mean relative amount for the control. Linear
254 regression with Pearson correlation coefficients were used to compare the agreement between the
255 RNA-Seq and qPCR results. For the additional HH, KO and MO populations, significant
256 differential expression between treatments was assessed using the log-transformed raw relative
257 expression values and a one tailed t-test.

258 *Population genetics and phylogeography of* Micrarchus

259 Contigs corresponding to all 13 mitochondrial protein coding genes were extracted from
260 the three RNA-Seq assemblies by tBLASTx sequence searching against the complete *D.*
261 *melanogaster* mitochondrial genome (DMU37541). The contigs were subsequently aligned and
262 pairwise similarities calculated. To generate SNP data to compare between the three populations,

263 a new Trinity assembly was constructed with the cleaned reads from all 24 individuals with the
264 same parameters as previously described. Individual clean reads were then mapped onto the new
265 combined assembly using Bowtie 2 (Langmead & Salzberg 2012). SNPs were subsequently
266 called from the Bowtie 2 mapped reads using the recommended default parameters for *mpileup*
267 in SAMtools v. 0.1.18 (Li *et al.* 2009). The data set was trimmed to only retain bialleic, unlinked
268 and high quality (Q > 30) SNPs with no missing data using VCFtools v. 0.1.10 (Danecek *et al.*
269 2011). Population structure was assessed by way of a principle component analysis (PCA)
270 performed in R (R Development Core Team 2012) and ancestry inference using ADMIXTURE
271 v.1.22 (Alexander *et al.* 2009) with K = 2 to K = 6.

272

273

274 **Results**

275 *Transcriptome assembly*

276 A total of 235 million 50 bp single-end Illumina reads were obtained from 24
277 individually-tagged cDNA libraries. Raw data have been submitted to the NCBI Sequence Read
278 Archive (SRA). An average of 9.8 million reads (range: 6.6-13.2 million reads) were generated
279 for each library (Table S4), with 99% of the data remaining after trimming. Cleaned reads were
280 *de novo* assembled by species and population: *M.* nov. sp. 2 (SP), *M.* nov. sp. 2 (MA) and *M.*
281 *hystriculeus* (PA); producing 42,425 (41,771 unigenes), 58,743 (57,576 unigenes) and 37,814
282 (37,334 unigenes) contigs, respectively (Table S4). Between 27% and 30% of the contigs within
283 each assembly were annotated by BLASTx against the *nr* database, with the species distribution
284 of top-matches overlapping among the three assemblies (Fig. S1). The distribution of high level
285 GO terms from the SwissProt BLASTx matches was extremely similar among the three
286 assemblies (Fig. S2).

287 *Phylogeography and population genetics*

288 A total of 210 individuals were sequenced, resulting in alignments of 530 bp for 28S and 1515
289 bp of COI/COII. All sequences have been submitted to NCBI Genbank. The 28S Bayesian and
290 likelihood phylogenies support each *Micrarchus* species as monophyletic, with *M. hystriculeus*
291 sister to *M.* nov. sp. 1, and *M.* nov. sp. 2 sister to *M.* nov. sp. 3 (Fig. 1; expanded phylogeny Fig.
292 S3). There is very little intraspecific variation at this locus, with between 98.5% and 100%
293 pairwise similarity between alleles from the same species. The COI/COII Bayesian phylogeny
294 does not support any *Micrarchus* species as monophyletic, with geographically-proximal
295 populations of different species often grouping together (Fig. 2; expanded phylogeny Fig. S4).

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

302 All *M.* nov. sp. 2 (MA) mtDNA protein coding sequences in the RNA-Seq data have a
303 greater pairwise nucleotide identity to those from *M. hystriculeus* (PA) (average pairwise identity
304 = 95.2%) than the conspecific *M.* nov. sp. 2 (SP) (average pairwise identity = 92.7%), apart from
305 *NADH dehydrogenase subunit 4L* (*ND4L*). The *ND4L* gene is 285 bp in *M.* nov. sp. 2 (MA) and
306 is 98.1% identical to *M. hystriculeus* (PA) (11 observed substitutions) and 98.6% identical to *M.*
307 nov. sp. 2 (SP) (ten observed substitutions). The degree of admixture in the nuclear genome
308 between *M. hystriculeus* and *M.* nov. sp. 2 was assessed using 45,785 biallelic unlinked
309 transcriptome wide SNPs extracted from all 24 individuals from three populations: 6 x *M.*
310 *hystriculeus* (PA) (Pure *M. hystriculeus* mtDNA); 6 x *M.* nov. sp. 2 SP (pure *M.* nov. sp. 2
311 mtDNA) and 12 x *M.* nov. sp. 2 (MA) (introgressed *M. hystriculeus* mtDNA). PCA analysis of
312 the SNP data identified three separate clusters that corresponded to the three populations (Fig. 3).
313 The first principle component explained 26.2% of the variation in the data and separates both *M.*
314 nov. sp. 2 populations from *M. hystriculeus*. The second principle component explained 14.6%
315 of the variation in the data and distinguishes the two *M.* nov. sp. 2 populations. Maximum
316 likelihood estimation was used to determine the ancestry of each individual from the SNP data,
317 with three being the optimal number of ancestral populations based on cross-validation errors
318 between different values of K (2-6). K = 3 clearly separates the three populations (Fig. 3). The

319 next best fit of K = 2 groups the populations into their respective species groups. Values of K
320 higher than three have a much lower fit to the data (higher cross-validation error). These data
321 demonstrate a high degree of differentiation in the nuclear genomes of the species of
322 *Micrarchus*, however, there is strong evidence of gene flow at mitochondrial DNA.

323 *Differential expression analysis*

324 Differentially-expressed transcripts as a result of cold-shock treatment were identified in
325 two alpine populations of *M.* nov. sp. 2 (MA and SP) and one lowland population of *M.*
326 *hystriculeus* (PA). Counts for differential expression analysis were generated by mapping
327 cleaned 50 bp Illumina HiSeq reads back onto their respective population transcriptome, with
328 between 84.3 and 92.9% (Mean = 89.5%; SD = 1.9%) of reads from each individual having at
329 least one valid alignment. Differential expression analyses identified 1774, 252 and 134
330 differentially expressed cold-responsive unigenes in *M.* nov. sp. 2 (SP), *M.* nov. sp. 2 (MA) and
331 *M. hystriculeus* (PA) populations, respectively (Table 1; Full details of the differentially-
332 regulated cold-responsive genes in Supplementary File B). A majority of the differentially-
333 expressed unigenes were unique to each population (*M.* nov. sp. 2 (MA) 52%; *M.* nov. sp. 2 (SP)
334 90%; *M. hystriculeus* (PA) 62%; Mean 68% ± 20%), sharing no BLASTx match (*E*-value
335 threshold = < $1e^{-10}$) with differentially-expressed genes identified in other populations (Fig. 4).
336 Cold-responsive unigenes that did overlap between populations were regulated in similar and
337 opposing directions (Fig. 4; Full details of correspondence between differentially-regulated cold-
338 responsive transcripts in Table S5). No unigene was universally up-regulated, or universally
339 down-regulated as a result of cold-shock in all three populations.

340 Enrichment of biological function GO terms in the differentially expressed unigenes
341 compared to the non-differentially expressed unigenes was assessed using a Fisher's exact test
342 (FDR <0.05). In *M.* nov. sp. 2 (SP) 32 molecular functions were significantly enriched in the
343 differentially expressed unigenes (Table 2). In *M.* nov. sp. 2 (MA) 3 molecular functions were
344 significantly enriched in the differentially expressed unigenes (Table 2). In *M. hystriculeus* (PA)
345 a single molecular function was significantly enriched in the differentially expressed unigenes
346 (Table 2). The GO terms for the structural constituent of cuticle (GO:0042302), structural
347 constituent of chitin-based cuticle (GO:0005214) and structural molecule activity (GO:0005198)
348 were significantly enriched in both *M.* nov. sp. 2 (SP) and *M.* nov. sp. 2 (MA).

349 *qPCR verification*

350 To validate the RNA-Seq results and to further explore geographical patterns of
351 differential expression, expression was also measured in a subset of six genes (Table S2) by
352 qPCR for technical (same individuals and RNA extractions for both methods) and biological
353 replicates (different individuals from the same population) in all three populations. Overall, there
354 was a strong correlation between the RNA-Seq and qPCR results for the technical replicates (*P*-
355 value < 0.001; Fig. 5; Fig. S5). There was also a significant positive correlation between the
356 RNA-Seq and qPCR results for the biological replicates (*P*-value < 0.009; Fig. 5, Fig. S5). The
357 expression of the qPCR candidates was also assessed in alpine *M.* nov. sp. 2 (MO) and *M.* nov.
358 sp. 2 (HH), and lowland *M. hystriculeus* (KO) (Fig. 5) to obtain further information on
359 intraspecific patterns of gene expression. There was a high level of intra- and inter-specific
360 variation in the magnitude of expression response to the cold-shock treatment. For example, in
361 *Endocuticle structural glycoprotein db-2* (*Cud2*) the direction of regulation in the additional
362 alpine *M.* nov. sp. 2 populations were consistent with *M.* nov. sp. 2 (MA), but in only *M.* nov. sp.

363 2 (HH) was the gene significantly down-regulated as a result of cold-shock. *Sarcosine*
364 *dehydrogenase* (*Sardh*) is significantly up-regulated in *M.* nov. sp. 2 (HH), but not in *M.* nov. sp.
365 2 (MO); even though the overall fold-change is higher in the latter. *Cathepsin L* is significantly
366 up-regulated in three of the four alpine populations (*M.* nov. sp. 2 (MA), *M.* nov. sp. 2 (SP) and
367 *M.* nov. sp. 2 (MO)). *Unannotated contig8923* (*Comp8923*) is up-regulated as a result of cold-
368 shock in both populations of the alpine species.

369

370 **Discussion**

371 All *Micrarchus* experience sub-zero temperatures in their respective environments. However the
372 frequency and duration of cold stress, and the minimum temperatures encountered by *M.* nov. sp.
373 2 are more extreme than experienced by its congeners (Salmon 1991; Dennis *et al.* 2013). Thus,
374 we expect *M.* Nov. sp. 2 to have a more cold-hardy genotype. In the context of the likely
375 restricted gene flow imposed by the sky island distribution of this species, we also expect local
376 adaptation of the gene expression response to low temperature. In this study we show that, across
377 a relatively small spatial scale, reduced gene flow and possible local adaptation are associated
378 with location-specific transcriptional responses to low temperature exposure.

379 *Genetic divergence and introgression among* Micrarchus *species and populations*

380 Our phylogeographic data clearly show strong differentiation of the nuclear genomes of
381 *Micrarchus* species and populations within *M.* nov. sp. 2, a prerequisite for local adaptation. All
382 species are monophyletic at 28S, and transcriptome wide SNPs clearly able to discriminate
383 populations and species. However, this resolution is lost in the mitochondrial genome, with
384 evidence of introgression of *M. hystriculeus* mtDNA into the other three *Micrarchus* species
385 (Fig. 2). The consistent phylogenetic clustering of geographically-proximate populations from
386 different *Micrarchus* species supports our conclusion that mtDNA replacement is due to
387 introgression rather than incomplete lineage sorting (Holder *et al.* 2001). Furthermore,
388 introgression is commonly observed in stick insects, including New Zealand species (Morgan-
389 Richards & Trewick 2005; Andersen *et al.* 2006; Buckley *et al.* 2008; Schwander & Crespi
390 2009). Several processes in insects may give rise to the complete mitochondrial capture with
391 reduced nuclear introgression that is witnessed in *M.* nov. sp. 2 from Mount Arthur (reviewed by

392 Toews & Brelsford 2012), including: (i) hybrid zone movement, (ii) sex-biased asymmetries, 393 (iii) adaptive introgression, (iv) demographic disparities and (v) *Wolbachia* infection. However, 394 further work is required to identify the cause of mtDNA introgression in *Micrarchus*. Reduced 395 nuclear introgression may indicate local adaptation in *M.* nov. sp. 2, with selection against 396 maladapted *M. hystriculeus* nuclear genes in *M.* nov. sp. 2 populations (Nosil *et al.* 2005).

397 *Cold-induced changes to transcription in* Micrarchus

398 The *de novo* assembled transcriptomes of the three *Micrarchus* populations were broadly 399 similar with analogous GO annotation and BLAST match frequencies within and between 400 species (Figs S1 & S2). However, extensive variation in the transcriptional response to cold-401 shock mirrors the genetic diversity witnessed in *Micrarchus*, with 68% (± 20%) of differentially-402 expressed unigenes being location specific. The most notable difference in transcriptional 403 response between these two species is the differential expression of cuticle-related unigenes in 404 alpine *M.* nov. sp. 2, but not in lowland *M. hystriculeus*. In the two genetically distinct and 405 geographically isolated populations of *M.* nov. sp. 2 used for RNA-Seq, structural cuticle 406 unigenes are predominately differentially regulated in opposite directions. Geographically-407 isolated populations of marine copepods show similar disparate responses to thermal stress in 408 genes associated with cuticle structure (Schoville *et al.* 2012). Structural reorganisation of the 409 cuticle in response to cold-shock in *M.* nov. sp. 2 may be an adaptation that has facilitated the 410 colonisation of the alpine environment and the development of a more cold-hardy phenotype.

411 At the individual cuticle unigene level, both *M.* nov. sp. 2 (MA) and *M.* nov. sp. 2 (SP) 412 up-regulate two *cathepsin L* (*Catl*) orthologous as a result of cold-shock. *Catl* is a proteolysis 413 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

437 replacement from the lowland *M. hystriculeus*. This provides some evidence for local adaptation
438 in *M.* nov. sp. 2, with selection against maladapted introgresing *M. hystriculeus* nuclear genes.
439 Small population sizes and extremely low migration rates increase the chance of fixation of
440 locally adapted traits that have even relatively small positive selective coefficients (Lenormand
441 2002; Kawecki & Ebert 2004). This raises the possibility that some of the fixed expression
442 differences between *M.* nov. sp. 2 populations are a result of local adaptation.

443 While the 'core' transcriptional response of cuticular gene expression has been
444 maintained, the variation in low-temperature response may mean that populations of *M.* nov. sp.
445 2 will respond differently to future altered climate regimes. This is contrary to genetically-
446 homogenous species where high dispersal between populations will prevent the maintenance of
447 local alleles and lead to a more uniform response (Case & Taper 2000). The evolution of
448 divergent transcriptional responses to low-temperature exposure in isolated populations of *M.*
449 nov. sp. 2 has two important implications. First, this divergence has evolved in response to
450 presumably similar montane environmental stressors from a common genetic background, which
451 implies that these two populations have solved the physiological challenges of montane habitats
452 in different ways. Thus, we show that there may be significant evolutionary divergence in the
453 responses to abiotic stressors even when the environmental stressors are ostensibly similar.
454 Second, local adaptation in response to environmental stressors presents challenges for managing
455 populations in the face of climate change, because locally-adapted genotypes may be unsuitable
456 for translocation to new locations, and may even perform poorly as their current habitat changes.
457 While such local adaptation has been identified in organismal-level studies (e.g. Pelini *et al.*
458 2009), our work suggests that it may be possible to identify functional local adaptation by
459 screening gene expression responses to simple abiotic stressors.

460 **Acknowledgements**

461 We would like to thank Jacob Corcoran for the guidance with qPCR, Rich Leschen, Lucy Shield,
462 Shelly Myers, Litza Coello, Chris Dennis, Mhairi McFarlane, Ana Ramon-Laca and Clive
463 Williams for assistance with fieldwork, the Greymouth Department of Conservation office for
464 access to field sites, Katya Ruggiero and Steve Vander Hoorn for statistical support, Daniel
465 White and the New Zealand eScience Infrastructure (NeSI) high-performance computing
466 facilities and the staff at the Centre for eResearch at the University of Auckland, for
467 bioinformatic support. The project was funded by The Royal Society of New Zealand Marsden
468 Fund (LCR0901), the Allan Wilson Centre for Molecular Ecology and Evolution and core
469 funding for Crown Research Institutes from the Ministry of Business, Innovation and
470 Employment's Science and Innovation Group.

471

472

473

474

475 **References**

476 Alexander DH, Novembre J, Lange K (2009) Fast model-based estimation of ancestry in unrelated
477 individuals. *Genome research*, **19**, 1655–1664.

478 Anders S, Huber W (2010) Differential expression analysis for sequence count data. *Genome Biology*, **11**,
479 R106.

480 Andersen DH, Pertoldi C, Loeschcke V, Scali V (2006) Developmental instability, hybridization and
481 heterozygosity in stick insects of the genus *Bacillus* (Insecta; Phasmatodea) with different modes of
482 reproduction. *Biological Journal of the Linnean Society*, **87**, 249–259.

483 Buckley TR, Attanayake D, Park D *et al.* (2008) Investigating hybridization in the parthenogenetic New
484 Zealand stick insect *Acanthoxyla* (Phasmatodea) using single-copy nuclear loci. *Molecular*
485 *Phylogenetics and Evolution*, **48**, 335–349.

486 Buckley TR, Nylander JAA, Bradler S (2010) The phylogenetic placement and biogeographical origins of
487 the New Zealand stick insects (Phasmatodea). *Systematic Entomology*, **35**, 207–225.

488 Buckley TR, Palma RL, Johns PM *et al.* (2012) The conservation status of small or less well known
489 groups of New Zealand terrestrial invertebrates. *New Zealand Entomologist*, **35**, 137–143.

490 Carl J (1913) Phasmides nouveaux ou peu connus du Muséum de Genève. *Revue Suisse de Zoologie*, **21**,
491 1–57.

492 Carrasco MA, Buechler SA, Arnold RJ *et al.* (2011) Elucidating the biochemical overwintering
493 adaptations of larval *Cucujus clavipes puniceus*, a nonmodel organism, via high throughput
494 proteomics. *Journal of Proteome Research*, **10**, 4634–4646.

495 Case TJ, Taper ML (2000) Interspecific competition, environmental gradients, gene flow, and the
496 coevolution of species' borders. *The American Naturalist*, **155**, 583–605.

497 Chown SL, Terblanche JS (2006) Physiological diversity in insects: Ecological and evolutionary contexts.
498 *Advances in Insect Physiology*, **33**, 50–152.

499 Clark M, Thorne M, Purac J *et al.* (2009) Surviving the cold: Molecular analyses of insect cryoprotective
500 dehydration in the Arctic springtail *Megaphorura arctica* (Tullberg). *BMC Genomics*, **10**, 328.

501 Colinet H, Nguyen TTA, Cloutier C, Michaud D, Hance T (2007) Proteomic profiling of a parasitic wasp
502 exposed to constant and fluctuating cold exposure. *Insect Biochemistry and Molecular Biology*, **37**,
503 1177–1188.

504 Conesa A, Götz S, García-Gómez JM *et al.* (2005) Blast2GO: A universal tool for annotation,
505 visualization and analysis in functional genomics research. *Bioinformatics*, **21**, 3674–3676.

506 Dahlhoff EP, Rank NE (2000) Functional and physiological consequences of genetic variation at
507 phosphoglucose isomerase: Heat shock protein expression is related to enzyme genotype in a
508 montane beetle. *Proceedings of the National Academy of Sciences of the United States of America*,
509 **97**, 10056–10061.

510 Danecek P, Auton A, Abecasis G *et al.* (2011) The variant call format and VCFtools. *Bioinformatics*, **27**,
511 2156–2158.

512 Denlinger DL, Lee RE (2010) *Low temperature biology of insects*. Cambridge, UK: Cambridge
513 University Press.

514 Dennis AB, Dunning LT, Dennis C, Sinclair BJ, Buckley TR (2013) Overwintering in New Zealand stick
515 insects. *New Zealand Entomologist*.

516 Dirnböck T, Essl F, Rabitsch W (2011) Disproportional risk for habitat loss of high-altitude endemic
517 species under climate change. *Global Change Biology*, **17**, 990–996.

518 Drummond AJ, Ashton B, Buxton S *et al.* (2012) Geneious v5.6, available form URL
519 http://www.geneious.com/.

520 Dunning LT, Dennis AB, Park D *et al.* (2013) Identification of cold-responsive genes in a New Zealand
521 alpine stick insect using RNA-Seq. *Comparative Biochemistry and Physiology Part D: Genomics*
522 *and Proteomics*, **8**, 24–31.

523 Dunning LT, Dennis AB, Thomson G *et al.* (2013) Positive selection in glycolysis among Australasian
524 stick insects. *BMC Evolutionary Biology*, **13**, 215.

525 Ghalambor CK, McKay JK, Carroll SP, Reznick DN (2007) Adaptive versus non-adaptive phenotypic
526 plasticity and the potential for contemporary adaptation in new environments. *Functional Ecology*,
527 **21**, 394–407.

528 Grabherr MG, Haas BJ, Yassour M *et al.* (2011) Full-length transcriptome assembly from RNA-Seq data
529 without a reference genome. *Nature biotechnology*, **29**, 644–652.

530 Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by
531 maximum likelihood. *Systematic Biology*, **52**, 696–704.

532 Hardcastle TJ, Kelly KA (2010) baySeq: Empirical Bayesian methods for identifying differential
533 expression in sequence count data. *BMC Bioinformatics*, **11**, 422.

534 Heald WF (1951) Sky islands of Arizona. *Natural History*, **60**, 56–63.

535 Hoffmann AA, Sørensen JG, Loeschcke V (2003) Adaptation of *Drosophila* to temperature extremes:
536 Bringing together quantitative and molecular approaches. *Journal of Thermal Biology*, **28**, 175–216.

537 Hoffmann AA, Willi Y (2008) Detecting genetic responses to environmental change. *Nat Rev Genet*, **9**,
538 421–432.

539 Holder MT, Anderson JA, Holloway AK (2001) Difficulties in detecting hybridization. *Systematic*
540 *Biology*, **50**, 978–982.

541 Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees.
542 *Bioinformatics*, **17**, 754–755.

543 Hurvich CM, Tsai C-L (1989) Regression and time series model selection in small samples. *Biometrika*,
544 **76**, 297–307.

545 Kawecki TJ, Ebert D (2004) Conceptual issues in local adaptation. *Ecology Letters*, **7**, 1225–1241.

546 Kingsolver JG (1989) Weather and the population-dynamics of insects: Integrating physiological and
547 population ecology. *Physiological Zoology*, **62**, 314–334.

548 Laayouni H, Garcia-Franco F, Chavez-Sandoval BE *et al.* (2007) Thermal evolution of gene expression
549 profiles in *Drosophila subobscura*. *BMC Evolutionary Biology*, **7**, 42.

550 Lalouette L, Williams CM, Hervant F, Sinclair BJ, Renault D (2011) Metabolic rate and oxidative stress
551 in insects exposed to low temperature thermal fluctuations. *Comparative Biochemistry and*
552 *Physiology Part A: Molecular & Integrative Physiology*, **158**, 229–234.

553 Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nature methods*, **9**, 357–
554 359.

555 Lenormand T (2002) Gene flow and the limits to natural selection. *Trends in Ecology and Evolution*, **17**,
556 183–189.

557 Li B, Dewey C (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a
558 reference genome. *BMC bioinformatics*, **12**, 323.

559 Li H, Handsaker B, Wysoker A *et al.* (2009) The sequence alignment/map format and SAMtools.
560 *Bioinformatics*, **25**, 2078–2079.

561 MacMillan HA, Williams CM, Staples JF, Sinclair BJ (2012) Reestablishment of ion homeostasis during
562 chill-coma recovery in the cricket *Gryllus pennsylvanicus*. *Proceedings of the National Academy of*
563 *Sciences of the United States of America*, **109**, 20750–20755.

564 Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads.
565 *EMBnet.Journal*, **17**, 10–12.

566 Morgan M, Anders S, Lawrence M *et al.* (2009) ShortRead: A bioconductor package for input, quality
567 assessment and exploration of high-throughput sequence data. *Bioinformatics*, **25**, 2607–2608.

568 Morgan-Richards M, Trewick SA (2005) Hybrid origin of a parthenogenetic genus? *Molecular Ecology*,
569 **14**, 2133–2142.

570 Nosil P, Vines TH, Funk DJ (2005) Reproductive isolation caused by natural selection against immigrants
571 from divergent habitats. *Evolution*, **59**, 705–719.

572 Oleksiak MF, Churchill GA, Crawford DL (2002) Variation in gene expression within and among natural
573 populations. *Nature genetics*, **32**, 261–266.

574 Overgaard J, Sørensen JG, Petersen SO, Loeschcke V, Holmstrup M (2005) Changes in membrane lipid
575 composition following rapid cold hardening in *Drosophila melanogaster*. *Journal of Insect*
576 *Physiology*, **51**, 1173–1182.

577 Pelini SL, Dzurisin JD, Prior KM *et al.* (2009) Translocation experiments with butterflies reveal limits to
578 enhancement of poleward populations under climate change. *Proceedings of the National Academy*
579 *of Sciences of the United States of America*, **106**, 11160–11165.

580 Polato NR, Voolstra CR, Schnetzer J *et al.* (2010) Location-specific responses to thermal stress in larvae
581 of the reef-building coral *Montastraea faveolata*. *PLoS ONE*, **5**, e11221.

582 Posada D (2008) jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution*, **25**,
583 1253–1256.

584 Purać J, Burns G, Thorne MAS *et al.* (2008) Cold hardening processes in the Antarctic springtail,
585 *Cryptopygus antarcticus*: Clues from a microarray. *Journal of Insect Physiology*, **54**, 1356–1362.

586 Qin W, Neal SJ, Robertson RM, Westwood JT, Walker VK (2005) Cold hardening and transcriptional
587 change in *Drosophila melanogaster*. *Insect Molecular Biology*, **14**, 607–613.

588 R Development Core Team (2012) R: A language and environment for statistical computing. Vienna,
589 Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org.

590 Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: A Bioconductor package for differential
591 expression analysis of digital gene expression data. *Bioinformatics*, **26**, 139–140.

592 Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models.
593 *Bioinformatics*, **19**, 1572–1574.

594 Salmon JT (1991) *The stick insects of New Zealand*. Reed Publishers, Auckland, New Zealand.

595 Schmieder R, Edwards R (2011) Quality control and preprocessing of metagenomic datasets.
596 *Bioinformatics*, **27**, 863–864.

597 Schmieder R, Lim YW, Edwards R (2012) Identification and removal of ribosomal RNA sequences from
598 metatranscriptomes. *Bioinformatics*, **28**, 433–435.

599 Schoville SD, Barreto F, Moy G, Wolff A, Burton R (2012) Investigating the molecular basis of local
600 adaptation to thermal stress: Population differences in gene expression across the transcriptome of
601 the copepod *Tigriopus californicus*. *BMC Evolutionary Biology*, **12**, 170.

602 Schwander T, Crespi BJ (2009) Multiple direct transitions from sexual to apomictic parthenogenesis in
603 *Timema* stick insects. *Evolution*, **63**, 84–103.

604 Sinclair BJ (2012) Variation in thermal performance among insect populations. *Physiological and*
605 *Biochemical Zoology*, **85**, 594–606.

606 Sinclair BJ, Roberts SP (2005) Acclimation, shock and hardening in the cold. *Journal of Thermal*
607 *Biology*, **30**, 557–562.

608 Sømme L (1995) *Invertebrates in hot and cold arid environments*. Springer-Verlag, Berlin.

609 Sørensen JG, Nielsen MM, Loeschcke V (2007) Gene expression profile analysis of *Drosophila*
610 *melanogaster* selected for resistance to environmental stressors. *Journal of Evolutionary Biology*,
611 **20**, 1624–1636.

612 Sugiura N (1978) Further analysts of the data by akaike' s information criterion and the finite corrections.
613 *Communications in Statistics - Theory and Methods*, **7**, 13–26.

614 Teets NM, Peyton JT, Ragland GJ *et al.* (2012) Combined transcriptomic and metabolomic approach
615 uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. *Physiological Genomics*,
616 **44**, 764–777.

617 Toews DPL, Brelsford A (2012) The biogeography of mitochondrial and nuclear discordance in animals.
618 *Molecular Ecology*, **21**, 3907–3930.

619 Vesala L, Salminen TS, Laiho A, Hoikkala A, Kankare M (2012) Cold tolerance and cold-induced
620 modulation of gene expression in two *Drosophila virilis* group species with different distributions.
621 *Insect Molecular Biology*, **21**, 107–118.

622 Wang H, Ma Z, Cui F *et al.* (2012) Parental phase status affects the cold hardiness of progeny eggs in
623 locusts. *Functional Ecology*, **26**, 379–389.

624 Weeks AR, Sgro CM, Young AG *et al.* (2011) Assessing the benefits and risks of translocations in
625 changing environments: a genetic perspective. *Evolutionary Applications*, **4**, 709–725.

626 Westwood JO (1859) Catalogue of orthopterous insects in the collection of the British Museum: Part I.
627 Phasmidae. Printed by order of the Trustees.

628 Ye J, Fang L, Zheng H *et al.* (2006) WEGO: A web tool for plotting GO annotations. *Nucleic Acids*
629 *Research*, **34**, W293–W297.

630 Zhang J, Marshall KE, Westwood JT, Clark MS, Sinclair BJ (2011) Divergent transcriptomic responses
631 to repeated and single cold exposures in *Drosophila melanogaster*. *The Journal of Experimental*
632 *Biology*, **214**, 4021–4029.

633 Zwickl DJ (2006) *Genetic algorithm approaches for the phylogenetic analysis of large biological*
634 *sequence datasets under the maximum likelihood criterion*. Unpublished Ph.D. dissertation, The
635 University of Texas at Austin, Texas, USA.

636

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.

659 **Figure Captions**

660 **Fig. 1:** Geographic distribution of the four *Micrarchus* species within New Zealand and their
661 evolutionary relationship based on 28S ribosomal RNA sequences. The 28S Bayesian phylogeny
662 is collapsed into the four species nodes, each with number of samples (*n*), number of different
663 haplotypes (*h*) and percentage pairwise identity between haplotypes (*i*) shown. Upper support
664 values are posterior probabilities estimated with MrBayes; lower support values are likelihood
665 bootstraps (100) estimated with GARLI. Scale bar represents the number of substitutions per
666 site. The full detail of each of the 43 sampling locations and expanded phylogeny is provided in
667 Table S1 and Fig. S3. Sites used for RNA-Seq and qPCR are named.

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

675 **Fig 3:** a) Principle components analysis and b) Inferred ancestry (Q-Plot) based on 45,785
676 biallelic unlinked SNPs from 24 individuals representing three populations and two *Micrarchus*
677 species (Paengaroa = *M. hystriculeus*; Mt. Arthur and Sewell Peak = *M.* nov. sp. 2). Each
678 individual in the Q-plot is represented by one vertical bar divided into varying proportions
679 (colours) representing ancestral populations (K). The optimal value of K is determined by the
680 lowest cross-validation procedure score (K = 3).

681 **Fig. 4:** Intra- and inter-specific variation in differentially-expressed cold-responsive loci from
682 three populations representing two species of *Micrarchus* stick insect. Each Venn diagram
683 represents a population and the number of its differentially expressed unigenes that have a
684 BLASTx (*E*-value $<1^{-10}$) sequence match with a differentially expressed unigenes in the other
685 populations. Cold-shock treatment = 21°C for 1 h; -1°C min-1 for 26 min; -5°C for 1 h; +1°C
686 min-1 for 26 min; 21°C for 1 h. Control treatment = 21°C for 3 h. If a unigene had more than one
687 BLASTx match only the match with the highest *E*-value was used.

688 **Fig. 5:** Heat map representing log transformed fold-changes in gene expression between control
689 (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}
690 for 26 min; 21°C for 1 h) *Micrarchus* stick insects using RNA-Seq and qPCR. * = Significantly
691 differentially expressed (qPCR samples t-test *P*-value < 0.05; RNA-Seq samples FDR < 0.05
692 edgeR or < 0.10 baySeq/DESeq. qPCR mRNA abundances normalised using two reference
693 genes (*pyruvate kinase* and *ATP synthase subunit beta*). Cartoons represent COI/COII and 28S
694 *Micrarchus* phylogenies, dotted line represents reticulated branch. Negative fold-changes
695 represent increased expression in treatment group (21°C for 1 h; -1°C min-1 for 26 min; -5°C for
696 1 h; +1°C min-1 for 26 min; 21°C for 1 h; positive fold-changes represent increased expression
697 in control group (21°C for 3 h). Inf = Infinite fold-change as a result of no recorded expression in
698 one sample.

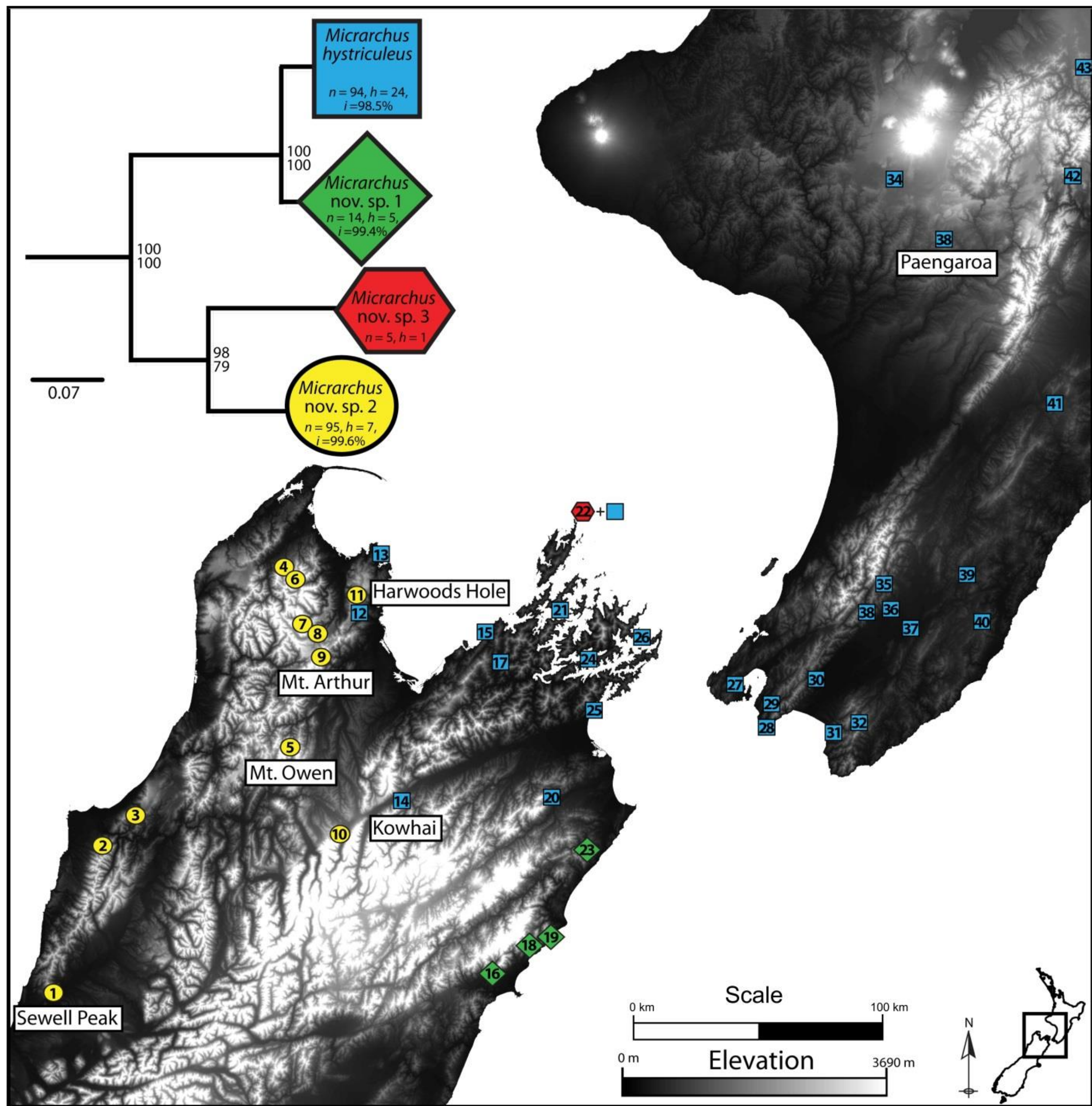
699

700

701

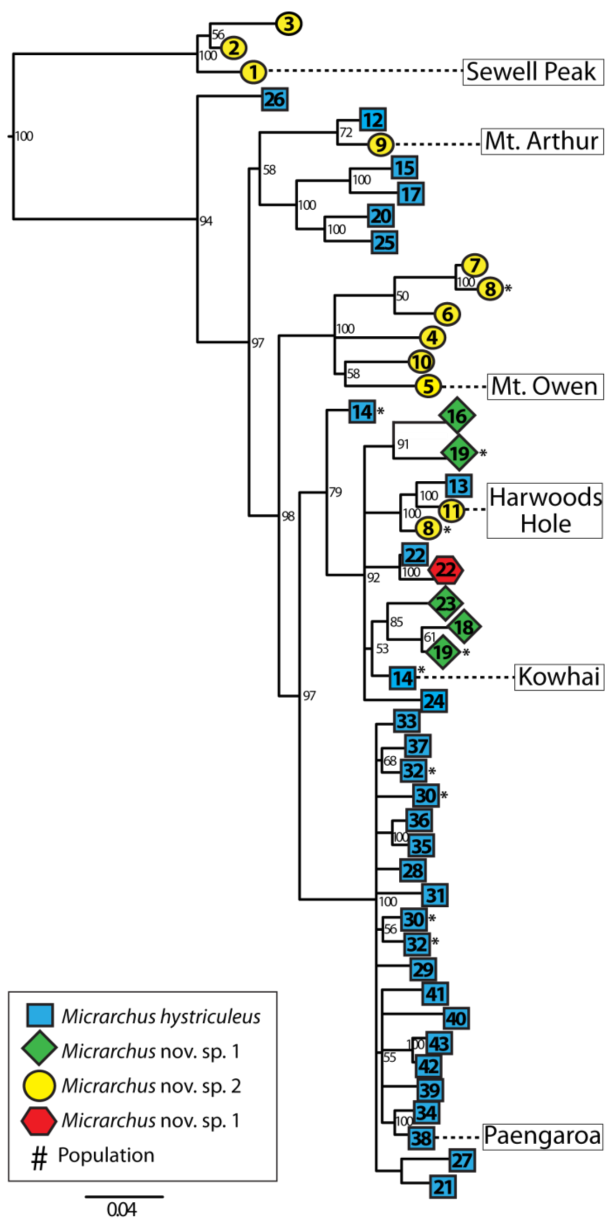
702

703 **Fig. 1**



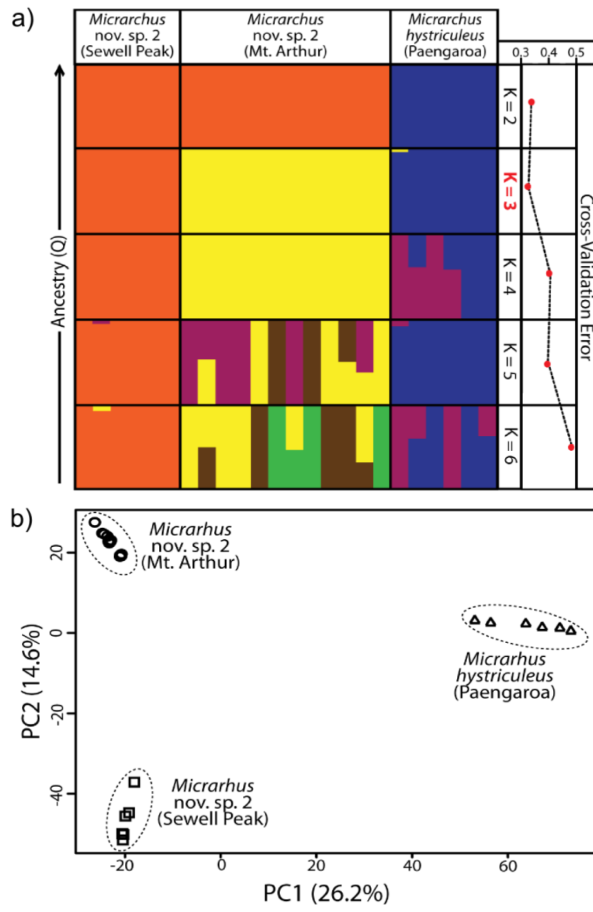
704

705 **Fig. 2**



706

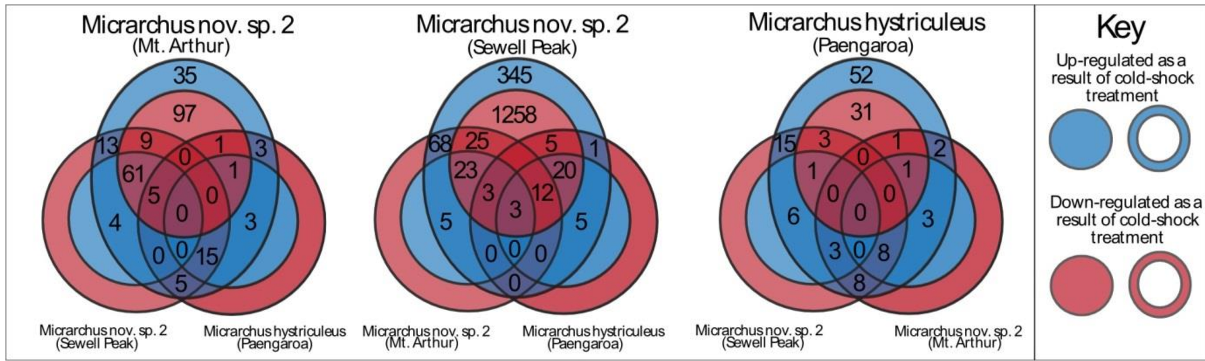
707 **Fig. 3**



708

709

710 **Fig. 4**

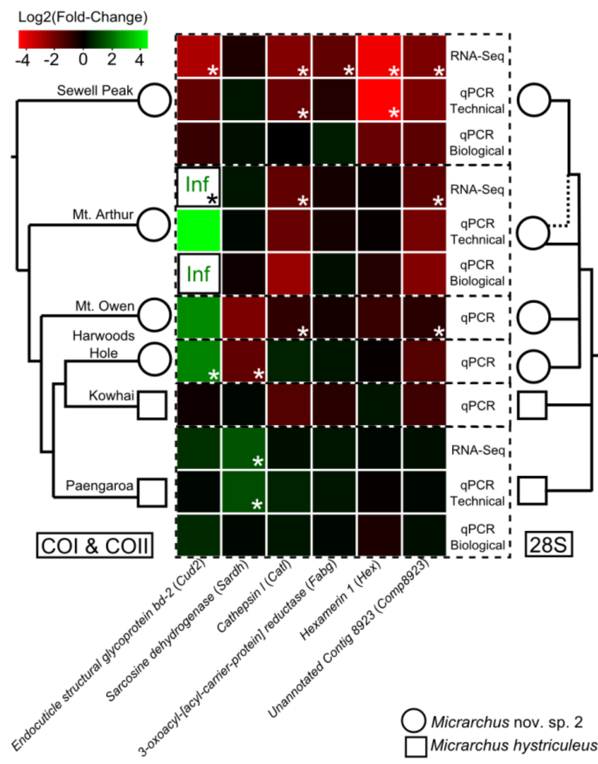


711

712

713

714 **Fig. 5**



715