Author/s:
McCulloch, GA;Guhlin, J;Dutoit, L;Harrop, TWR;Dearden, PK;Waters, JM

Title:
Genomic signatures of parallel alpine adaptation in recently evolved flightless insects

Date:
2021-12-01

Citation:
McCulloch, G. A., Guhlin, J., Dutoit, L., Harrop, T. W. R., Dearden, P. K. & Waters, J. M. (2021). Genomic signatures of parallel alpine adaptation in recently evolved flightless insects. Molecular Ecology, 30 (24), pp.6677-6686. https://doi.org/10.1111/mec.16204.

DR. GRAHAM A MCCULLOCH (Orcid ID : 0000-0003-1462-7106)
DR. LUDOVIC DUTOIT (Orcid ID : 0000-0002-0164-9878)
DR. JONATHAN WATERS (Orcid ID : 0000-0002-1514-7916)

Article type : Original Article

Genomic signatures of parallel alpine adaptation in recently-evolved flightless insects

McCulloch G.A.[1]*, Guhlin J.[2], Dutoit L.[1], Harrop T.W.R.[2], Dearden P.K.[2], Waters J.M.[1]

[1] *Department of Zoology, University of Otago. PO Box 56, Dunedin, 9054, New Zealand*
[2] *Genomics Aotearoa and Department of Biochemistry, University of Otago. PO Box 56, Dunedin, 9054, New Zealand*

* Corresponding author; email: graham.mcculloch@otago.ac.nz ; phone +64 3 479 4039

Running title: Repeated adaptation in upland stoneflies

Keywords: Plecoptera, stoneflies, wing loss, fecundity, incipient species, genotyping-by sequencing.

Abstract

Natural selection along elevational gradients has potential to drive predictable adaptations across distinct lineages, but the extent of such repeated evolution remains poorly studied for many widespread alpine taxa. We present parallel genomic analyses of two recently-evolved flightless alpine insect lineages to test for molecular signatures of repeated alpine adaptation. Specifically, we compare low-elevation versus alpine stonefly ecotypes from parallel stream populations in which flightless upland ecotypes have been independently derived. We map

67,922 polymorphic genetic markers, generated across 176 *Zelandoperla fenestrata* specimens from two independent alpine stream populations in New Zealand's Rock and Pillar Range, to a newly developed plecopteran reference genome. Genome-wide scans revealed 31 regions with outlier SNPs differentiating lowland versus alpine ecotypes in Lug Creek, and 37 regions with outliers differentiating ecotypes in Six Mile Creek. Of these regions, 13% (8/60) yielded outlier SNPs across both within-stream ecotype comparisons, implying comparable genomic shifts contribute to this repeated alpine adaptation. Candidate genes closely linked to repeated outlier regions include several with documented roles in insect wing-development (e.g. *dishevelled*), suggesting that they may contribute to repeated alpine wing reduction. Additional candidate genes have been shown to influence insect fecundity (e.g. *ovo*) and lifespan (e.g. *Mrp4*), implying that they might contribute to life history differentiation between upland and lowland ecotypes. Additional outlier genes have potential roles in the evolution of reproductive isolation among ecotypes (*hedgehog* and *Desaturase* 1). These results demonstrate how replicated outlier tests across independent lineages can potentially contribute to the discovery of genes underpinning repeated adaptation.

Introduction

The question of how species adapt to environmental heterogeneity has long fascinated researchers (Darwin, 1859). Biologists have long understood that taxa inhabiting similar environments often evolve similar phenotypes (Juan, Guzik, Jaume, & Cooper, 2010; Pan et al., 2019). Indeed, similar morphological and physiological adaptations across independent lineages — in response to comparable environmental shifts — can provide compelling evidence of the power of natural selection (Delgado, Górski, Habit, & Ruzzante, 2019; Dyer et al., 2012; Herman et al., 2018; Hoekstra, 2006; McCulloch et al., 2021; Veale & Russello, 2017) . Until recently, however, the extent of repetitive evolutionary shifts at the genomic level has remained difficult to assess for many taxa.

High-throughput sequencing approaches are revolutionising our understanding of repeated adaptation, as they allow researchers to identify genomic variants underpinning repeated morphological changes (Delgado et al., 2019; Herman et al., 2018; Matz, 2018; Nosil et al., 2018; Sackton & Clark, 2019). Several recent studies have demonstrated that repeated adaptation in some systems may arise completely independently via distinct *de novo* mutations across different populations (e.g. Gould & Stinchcombe, 2017; Hu et al., 2017; Pan

et al., 2019). By contrast, other analyses have shown that parallel selection on standing genetic variation often underpin similar evolutionary outcomes across distinct regions and populations (Terekhanova et al., 2014; Van Belleghem et al., 2018). Intriguingly, some recent analyses suggest that both *de novo* mutations and selection on standing variation can facilitate repetitive evolution within taxa over different spatial scales (Fang, Kemppainen, Momigliano, Feng, & Merilä, 2020; Härer, Bolnick, & Rennison, 2021; McCulloch et al., 2021; Waters & McCulloch, 2021).

Adaptive shifts in response to ecological variation may occur rapidly via selection on genes possessing a range of pleiotropic functions (Archambeault, Bärtschi, Merminod, & Peichel, 2020; Friedman, Twyford, Willis, & Blackman, 2015; Hohenlohe et al., 2010; Peichel & Marques, 2017). Additionally, such rapid adaptation may also be facilitated via selection on genes that are tightly linked in regions of low recombination (Gonzalez, Aramendia, & Davison, 2019; Ortiz-Barrientos, Engelstädter, & Rieseberg, 2016; Peichel & Marques, 2017; Roda, Walter, Nipper, & Ortiz-Barrientos, 2017; Todesco et al., 2020; Yeaman, Aeschbacher, & Bürger, 2016; Yeaman & Whitlock, 2011). Concerted adaptations across multiple linked traits can also directly facilitate speciation when such loci also influence reproductive divergence among lineages (Gavrilets, 2004). Such traits are typically referred to as 'magic traits' (in the case of pleiotrophic loci; Salis, Lorin, Laudet, & Frédérich, 2019; Servedio, Van Doorn, Kopp, Frame, & Nosil, 2011) or 'pseudomagic traits' (in the case of tightly clustered gene complexes; Estandia & Sendell-Price, 2020; Rundle & Nosil, 2005; Servedio & Bürger, 2020). While such genomic features are hypothesised to be relatively common in nature, the broader importance of these traits in facilitating speciation remains unclear (Servedio & Bürger, 2020; Servedio et al., 2011).

Organisms inhabiting harsh upland environments often evolve distinctive suites of characters to overcome the challenges of montane conditions (Billings, 1974; Hodkinson, 2005; Somme, 1989). High-elevation grassland habitats, for instance, are subject to high exposure and low temperatures, as well as reduced air pressure, and limited food availability relative to lowland (forested) ecosystems (Dillon, Frazier, & Dudley, 2006; Nagy & Grabherr, 2009). Recent studies suggest that strong winds, for instance, may play a particularly important role in selecting for wing reduction (Leihy & Chown, 2020; McCulloch, Foster, Ingram, & Waters, 2019b) which is a common feature of alpine insect assemblages (Roff, 1990). However, researchers are only beginning to understand the genetic bases of many adaptations

to harsh alpine conditions (Cheviron & Brumfield, 2012; Guo, Lu, Liao, & Merilä, 2016; Jackson et al., 2020; Montero-Mendieta et al., 2019).

Widespread dimorphic taxa represent strong systems for understanding the ecological genomic bases of adaptative shifts linked to environmental heterogeneity (Fang et al., 2020; Suzuki, Suzuki, & Tojo, 2019; Van Belleghem et al., 2018). The southern New Zealand stonefly *Zelandoperla fenestrata* complex, for instance, exhibits widespread dimorphic wing phenotypes linked to elevational variation (full-winged: lowland; vestigial-winged: upland; Figure 1a) (Dussex, Chuah, & Waters, 2016; McCulloch, Wallis, & Waters, 2009; McLellan, 1999). The tight association between wing length and the position of the alpine treeline in this species suggests that flight loss is driven by environmental conditions above the alpine treeline (e.g. high winds; Foster, McCulloch, Vogel, Ingram, & Waters 2021; McCulloch et al., 2019a). However, it is possible that wing loss may be an indirect consequence of selection on other traits associated with alpine adaptation (see Waters, Emerson, Arribas, & McCulloch, 2020). Recent genome-wide analyses of this complex indicate that wing-reduced upland ecotypes in each stream are genomically distinct from one another and from their corresponding flighted population (McCulloch et al., 2019a), and have evolved repeatedly from lowland source populations (Figure 1c, d; McCulloch et al. 2021). Indeed, repeated divergence of wing-reduced alpine ecotypes has arisen both locally (among independent stream populations; McCulloch et al., 2019a) and more broadly (among distinct mountain ranges; McCulloch et al., 2021).

Based on this evidence for repeated evolution of alpine ecotypes, we predict that independently-evolved flightless *Z. fenestrata* ecotypes should thus show evidence of repeated selection at genes associated with alpine adaptation. To test this hypothesis, we conduct parallel genomic analyses of genetically-divergent flightless ecotypes from separate streams in New Zealand's Rock and Pillar Range. We map SNPs to a newly annotated stonefly genomic assembly, and use outlier approaches to test for genomic convergence linked to repeated alpine adaptation.

Materials and Methods

Stonefly sampling and genotyping-by-sequencing

179 *Zelandoperla* specimens were sampled from two independent alpine stream populations in the Rock and Pillar Range, southern New Zealand (Six Mile Creek; Lug Creek; Figure 1)

as described by McCulloch et al. (2019a). Specimens were collected across a range of elevations within each stream (Table S1). Both streams display elevational clines in ecotype frequency, with full-winged ecotypes dominating lowland samples, and flightless ecotypes dominating alpine samples. Previous coalescent phylogeographic analyses of these populations indicate that each flightless ecotype was independently derived from the corresponding lowland flighted ecotype (McCulloch et al., 2021; McCulloch et al., 2019a). Details of stonefly sampling, rearing, phenotypic analysis, and genotyping-by-sequencing (GBS) details are provided in McCulloch et al. (2019a).

Genome annotation

We annotated the *Z. fenestrata* genome (hosted at https://www.genomics-aotearoa.org.nz/data) using funannotate v. 1.7.2 (Love et al., 2019). We trained with *funannotate train* using RNA-seq reads from McCulloch et al. (2019c). This created 21,429 PASA gene models (Haas et al., 2003). We then used *funannotate predict* with *fly* as the initial BUSCO seed species, and with the optimize_augustus parameters. We iterated the *predict* step a further two times to improve gene prediction, replacing *fly* with the newly created *Z. fenestrata* species for the remaining two steps. The predict step obtained 41,098 predictions from EVM (Haas et al., 2008). The removal of 4,776 primarily transposable elements left 36,322 gene models. Published files are available at https://www.genomics-aotearoa.org.nz/data. Scripts are available at: https://github.com/jguhlin/stonefly_anno.

Variant calling

Single nucleotide polymorphisms (SNPs) were extracted from the GBS dataset (NCBI Sequence Read Archive PRJNA530622) via Stacks 2.53 (Rochette, Rivera-Colón, & Catchen, 2019) using the *Z. fenestrata* reference genome v1 (McCulloch et al., 2021). The detailed SNP calling procedure and associated code can be found in Supplementary Appendix A. Briefly, reads were demultiplexed using the *process_radtags* module of Stacks. Reads were then mapped to the reference genome using the Burrows-Wheeler aligner implemented in BWA-MEM v0.7 (Li & Durbin, 2009). Mapped files were sorted and compressed using SAMtools v1.10 (Li et al., 2009). We used the *ref_align* pipeline of Stacks with the default parameters to call the SNPs. Following genotyping, we used the *populations* module of Stacks to further filter the data, retaining only SNPs that were genotyped in at least 75% of samples, following filtering optimised using similarly constructed dataset in McCulloch et al. (2021). Three samples were subsequently removed from the dataset, as

preliminary principal component analysis indicated that they were the wrong species, leaving 176 individuals in the final dataset (43 vestigial-winged from Lug, 44 full-winged from Lug, 47 vestigial-winged from Six Mile, 42 full-winged from Six Mile).

Linkage disequilibrium

We assessed linkage disequilibrium decay using the --geno-r2 option of VCFtools (Danecek et al., 2011). This analysis was conducted for full-winged and vestigial-winged ecotypes from each stream independently (i.e. four comparisons). Samples from different elevations were combined, as there is no geographic genetic structure within ecotypes in either of the streams (McCulloch et al., 2019a). In addition, we also estimated linkage disequilibrium decay for the complete data set (all 176 individuals included). After estimating r^2 for all the within-contig SNPs pairs, we randomly selected 100,000 SNP comparisons located within the same 100kb. A weighted regression was then fitted using a loess model (Cleveland, Grosse, & Shyu, 1992), as implemented in the base package stats for R (R Core Team, 2019). We determined the extent of linkage among SNPs in the genome by assessing when r^2 values reached background levels. This empirically-derived value was subsequently used when testing for shared outlier regions across the two streams, and for identifying genes closely linked to outlier SNPs (see below).

Outlier detection and analysis

The flightless alpine ecotypes inhabiting Six Mile and Lug Creek are recently and independently derived from the corresponding lowland flighted ecotype (McCulloch et al., 2021; McCulloch et al., 2019a). We therefore tested for SNPs significantly differentiated among the full-winged and vestigial-winged lineages in each stream, to identify genes potentially involved in alpine adaptation. We utilised two conceptually contrasting approaches to search for outlier SNPs: *BayeScan* 2.1 (Foll & Gaggiotti, 2008) and *pcadapt* v4.3.2 (Privé, Luu, Vilhjálmsson, & Blum, 2020). Prior to outlier detection, we removed uninformative SNPs by applying a minor allele frequency filter of 5%. Outlier SNPs were detected in *pcadapt* using a principal component analysis approach. The first principal component (K=1) was used for estimating the test statistics. We converted the *P* values outputted by *pcadapt* to *Q* values using the R package *qvalue* (Storey, Bass, Dabney, & Robinson, 2020). We tested for outlier loci strongly differentiating ecotypes using *BayeScan*, with the default parameters used. We pooled samples from different elevations together for this analysis, as no geographic genetic structure has been detected within ecotypes within

these streams. SNPs with a Q value < 0.05 using either *pcadapt or BayeScan* were considered as potential outliers.

As the full-winged and vestigial-winged ecotypes in each stream are genomically distinct (McCulloch et al., 2021; McCulloch et al., 2019a; Fig. 1c), it is possible that outliers detected in individuals within individual stream comparisons may be the results of neutral processes (e.g. genetic drift), rather than positive selection. We therefore focused primarily on outlier regions shared across both independent stream comparisons, as these parallel shifts are unlikely to be the result of such drift. Although repeated evolution may be the result of parallel selection on standing genetic variation ("repeated sorting"; Magalhaes et al., 2021; Waters & McCulloch, 2021), independent *de novo* mutations in the same genomic regions may also lead to repeated morphological shifts (e.g. Gould & Stinchcombe, 2017). If adaptation were underpinned by *de novo* mutations, we would expect to find different SNPs linked to these mutations across distinct stream populations. Therefore, in addition to looking for outlier SNPs shared among streams, we also tested for shared outlier regions. We considered outlier SNPs to be in a shared outlier region if they were located within 20kb of one another (based on the extent of linkage disequilibrium among makers in this species; see results).

We estimated the number of 'potential outlier regions' in our analysis by combining all SNPs within 20kb of one another into regions, and then calculating how many of these regions there were. These 'potential outlier regions' could potentially extend beyond 20kb if three or more SNPs were involved. We used Fisher's exact test to determine whether there were more shared SNPs among streams, or more shared outlier regions among streams, than would be expected by chance. We plotted allele frequencies of outlier SNPs against elevation, to compare their clinal distributions relative to background SNPs.

We used the *Z. fenestrata* reference genome to search for candidate alpine adaptation genes within 20kb of outlier SNPs. Putative functions of such genes were determined using the UniProtKB database (https://www.uniprot.org/). In addition to focusing on genes potentially associated with flight loss, we also searched for genes potentially associated with additional alpine adaptations, including potential olfactory shifts (Neupert, McCulloch, Foster, Waters, & Szyszka, 2020), increased body size (McCulloch & Waters, 2018a), delayed emergence (McCulloch & Waters, 2018b), increased longevity (McLellan, 1999), increased fecundity

(Guerra, 2011), as well as genes that might potentially be involved in reproductive isolation among sympatric ecotypes (e.g. sexual development; Kroos, Waters, & McCulloch, 2021; McCulloch et al., 2021; McCulloch et al., 2019a).

Results

Our GBS analyses of 176 *Z. fenestrata* specimens from the Rock and Pillar Range identified 67,922 SNPs mapped across 2,275 contigs. After removing SNPs with minor allele frequencies of <0.05, we retained 21,496 SNPs mapped across 1,932 contigs. Linkage disequilibrium was similar across all groups, with r^2 values reaching background levels at distances greater than 20kb (Figure S1-S3).

BayeScan tests for outlier SNPs significantly differentiated between full-winged and wing-reduced ecotypes within streams identified 124 outlier SNPs across 63 regions within the Six Mile Creek population (Table S2), and 48 outlier SNPs across 35 regions in the Lug Creek population (Table S3). Crucially, ten of these regions contained significant outlier SNPs in *both* the Six Mile and Lug among-ecotype comparisons (Figure 2a, Table 1). By comparison, *pcadapt* analyses identified 195 outlier SNPs differentiating ecotypes across 107 regions within the Six Mile population (Table S4), and 309 outliers across 176 regions in Lug Creek (Table S5). Seventeen of these regions contained significantly differentiated SNPs in both the Six Mile and Lug Creek among-ecotype comparisons (Figure 2b). The majority (8/10) of outliers detected across both streams using *BayeScan* were also detected with *pcadapt* (Figure S4; Table 1).

In combination, the two outlier tests revealed 19 regions containing significant outlier SNPs for both Six Mile and Lug ecotype comparisons (Table 1), and almost half (8/19) of these outlier regions were detected in both populations for both methods (Figure S4; Table 1). In some cases, the same outlier SNP was significantly differentiated across both Lug and Six Mile ecotype comparisons (e.g. on contig 192), with a Fisher's exact test indicating that there were significantly more shared outlier SNPs among streams than would be expect by chance ($P = 0.0034$). In these cases, the nucleotide shifts among ecotypes occurred in the same direction in both streams. In other cases, *different* SNPs within the same outlier region were detected in each stream (e.g. on contig 6423). In many of these situations, the same allelic shift was evident across both ecotype comparisons, but this change was only statistically significant in one of the streams. By contrast, in almost half these cases (15/34; 44%) the

outlier SNPs were detected in only one of stream ecotype comparisons, perhaps consistent with *de novo* mutation within a single stream.

Outlier SNPs were detected at one or both streams by both *pcadapt* and *BayeScan* across 60 distinct outlier regions. Overall, although outlier SNPs were detected at only a small proportion (1.5%; 60/3,908) of regions examined, a significant number (13%; 8/60) of these outlier regions showed differentiation among ecotypes for *both* Lug and Six Mile populations ($P = 2.0 \times 10^{-10}$; Figure 2c). Overall, these findings suggest that selection has occurred repeatedly at similar parts of the genome across these geographically independent ecotype comparisons. We identified concordant elevational clines in the allelic frequencies of outlier SNPs shared across Six Mile Creek and Lug Creek (Figure 3). No such patterns were evident for non-outlier SNPs (i.e. SNPs that are not under selection; Figure 3).

Almost half of the outlier regions detected by either *BayeScan* or *pcadapt* (9/19) were located within 20kb of at least one annotated gene (Table 1). Fourteen outlier SNPs were located directly within a gene, with five of these SNPs in introns, versus nine in coding regions. The majority of the SNPs in noncoding regions (8/9) caused synonymous mutations, but the SNP in contig 192 caused a nonsynonymous mutation. Half (6/12) of the genes linked to outliers in both Six Mile and Lug Creek populations have functions potentially associated with documented phenotypic differentiation between alpine (flightless) and lowland (flighted) ecotypes (Table 1). For instance, several of the identified genes have previously been associated with insect wing development, including *hedgehog* (*hh*) and *disheveled (dsh)*. Several additional genes tightly linked to outlier SNPs have known roles in determining additional characteristics that differ between upland and lowland ecotypes, including insect fecundity (e.g. *ovo*) and mating behaviour (e.g. *Desaturase 1* (*Desat1*)). Several of the outlier SNPs associated with ecotype differentiation in only a single stream were also linked to genes with potential roles in upland adaptation (Table S6, S7).

Discussion

Our genome-wide analyses identified a number of genes potentially involved in repeated alpine adaptation across two independently-evolved upland *Z. fenestrata* lineages. Crucially, a significant proportion of outlier genomic regions (>10%) were strongly differentiated across both independent ecotype comparisons. These findings imply that natural selection has acted repeatedly on similar regions of the genome in these distinct alpine populations,

suggesting repetitive adaptation has produced similar evolutionary outcomes across multiple lineages (e.g. Hill et al., 2019; Jones et al., 2012; Soria-Carrasco et al., 2014; Van Belleghem et al., 2018; Villoutreix et al., 2020). While it is possible that some of these shared outlier regions may have non-adaptive explanations (e.g. background selection; see Hoban et al., 2016; Matthey-Doret & Whitlock, 2019), the over-representation of genes with potential roles in alpine adaptation implies a substantial role for positive selection. Future functional validation of these candidate genes will nevertheless be needed to confirm their direct role in alpine adaptation (see Wolf & Ellegren, 2017).

Several of the identified genes closely linked to outlier SNPs have key roles in insect wing development. For instance, for both Lug and Six Mile alpine ecotypes we detected outlier SNPs in close proximity to the important insect developmental gene *hedgehog*, which has a key role in specifying cell-fate (Ingham & McMahon, 2001). Intriguingly, this gene is also strongly differentially expressed in the wing buds of full-winged versus vestigial-winged *Z. fenestrata* ecotypes (McCulloch et al., 2019a), suggesting it may indeed contribute to wing loss in this system. *Hedgehog* has also been shown to influence insect sexual development (Chen, Christiansen, & Baker, 2005; Kopp, 2012; Price, Egizi, & Fonseca, 2015). It is therefore conceivable that mutations at this pleiotropic developmental gene might influence both wing-reduction and reproductive compatibility among *Zelandoperla* ecotypes, ultimately underpinning the ecological speciation of these diverging lineages (McCulloch et al., 2021).

Desaturase 1, another gene closely linked to outlier SNPs across both stream populations, plays an important role in the production and perception of sex pheromones in *Drosophila* (Bousquet & Ferveur, 2012; Bousquet et al., 2012). This gene is also involved in free-flight odor tracking in *Drosophila* (Houot et al., 2018), and thus has potential to influence flight behaviour (Houot, Gigot, Robichon, & Ferveur, 2017). It is therefore conceivable that mutations at this gene could be linked to changes in both flight ability and reproductive compatibility among ecotypes of the *Z. fenestrata* complex (Kroos et al., 2021; McCulloch et al., 2021). Ideally, the potential role of parallel outlier regions detected in the current study should be validated more widely across the range of the *Z. fenestrata* complex, including additional regions where alpine and lowland ecotypes co-occur, to shed further light on their possible contributions to both alpine adaptation and speciation processes.

Although our study tested for outliers across a large suite of SNPs, the total number of markers examined covers only a fraction of the genome. It is therefore likely that the effects of many genes associated with alpine adaptation remain undetected in our study (see Catchen et al., 2017; Lowry et al., 2017). Furthermore, while we detected several shared outlier loci differentiating ecotypes across the two independent populations, a high proportion of outlier SNPs were unique to one or other populations. It is possible that some of the outlier SNPs detected within individual streams may have diverged via neutral processes (genetic drift) rather than through selection.

The proportion of outlier regions shared across independently-diverged populations in the current study (>10%) exceeds the proportions of parallel-divergent loci reported by comparable studies from some comparable repetitive adaptation systems (e.g. Feulner & Seehausen, 2019; Salisbury et al., 2020). We suggest that the close physical proximity of our populations (parallel mountain streams just a few km apart, exhibiting similar selective regimes) may contribute to this relatively high proportion of shared outlier genomic regions. Furthermore, the ancestral lowland (full-winged) *Z. fenestrata* populations (from which the vestigial-winged upland lineages were derived; Figure 1) share very similar genetic backgrounds (McCulloch et al., 2019a). This recent shared ancestry is likely to help fuel such repeated adaptive processes, given that replicated evolutionary processes often arise via repeated selection on standing genetic variation (e.g. Haenel, Roesti, Moser, MacColl, & Berner, 2019; Lai et al., 2019; Van Belleghem et al., 2018). Our results suggest that the genetic background of lineages subject to similar selective regimes may play an influential role in the determining the extent of genomic parallelism associated with such processes (Delgado et al., 2020; Fang et al., 2020). Interestingly, in many cases, different outlier SNPs within the same outlier region were identified across the two stream populations. While these results might, to some extent, be an artefact of missing data that commonly affects GBS analyses (see Hoban et al., 2016), they might alternatively indicate that both sorting of standing variation and independently derived *de novo* mutations have combined to facilitate repeated adaptation across these closely related populations (Waters & McCulloch, 2021).

Broadly, our analysis provides evidence that parallel selection can lead to repeated divergence across the same genomic regions in independent alpine lineages. Future whole-genome resequencing analyses promise to resolve in more detail the genomic dynamics of these intriguing repetitive evolutionary processes.

Acknowledgements

This work was supported by Marsden contracts UOO1412 and UOO2016 (Royal Society of New Zealand).

References

Archambeault, S. L., Bärtschi, L. R., Merminod, A. D., & Peichel, C. L. (2020). Adaptation via pleiotropy and linkage: Association mapping reveals a complex genetic architecture within the stickleback Eda locus. *Evolution Letters, 4*, 282-301.

Billings, W. D. (1974). Adaptations and origins of alpine plants. *Arctic and Alpine Research*, 129-142.

Bousquet, F., & Ferveur, J.-F. (2012). desat1: A Swiss army knife for pheromonal communication and reproduction? *Fly, 6*, 102-107.

Bousquet, F., Nojima, T., Houot, B., Chauvel, I., Chaudy, S., Dupas, S., . . . Ferveur, J.-F. (2012). Expression of a desaturase gene, desat1, in neural and nonneural tissues separately affects perception and emission of sex pheromones in *Drosophila*. *Proceedings of the National Academy of Sciences, 109*, 249-254.

Catchen, J. M., Hohenlohe, P. A., Bernatchez, L., Funk, W. C., Andrews, K. R., & Allendorf, F. W. (2017). Unbroken: RADseq remains a powerful tool for understanding the genetics of adaptation in natural populations. *Molecular Ecology Resources, 17*, 362-365.

Chen, E. H., Christiansen, A. E., & Baker, B. S. (2005). Allocation and specification of the genital disc precursor cells in *Drosophila*. *Developmental Biology, 281*, 270-285.

Cheviron, Z., & Brumfield, R. (2012). Genomic insights into adaptation to high-altitude environments. *Heredity, 108*, 354-361.

Cleveland, W., Grosse, E., & Shyu, W. (1992). *Statistical models in S* (C. J. M. & H. T. J. Eds.). Pacific Grove, CA: Wadsworth & Brooks/Cole.

Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., . . . Sherry, S. T. (2011). The variant call format and VCFtools. *Bioinformatics, 27*, 2156-2158.

Darwin, C. (1859). *On the origin of the species by natural selection*. London: John Murray.

Delgado, M. L., Górski, K., Habit, E., & Ruzzante, D. E. (2019). The effects of diadromy and its loss on genomic divergence: The case of amphidromous *Galaxias maculatus* populations. *Molecular Ecology, 28*, 5217-5231.

404 Delgado, M. L., Manosalva, A., Urbina, M. A., Habit, E., Link, O., & Ruzzante, D. E. (2020).
405 Genomic basis of the loss of diadromy in *Galaxias maculatus*: insights from
406 reciprocal transplant experiments. *Molecular Ecology, 2020*, 4857-4870.
407 Dillon, M. E., Frazier, M. R., & Dudley, R. (2006). Into thin air: physiology and evolution of
408 alpine insects. *Integrative and Comparative Biology, 46*, 49-61.
409 Dussex, N., Chuah, A., & Waters, J. M. (2016). Genome-wide SNPs reveal fine-scale
410 differentiation among wingless alpine stonefly populations and introgression between
411 winged and wingless forms. *Evolution, 70*, 38-47.
412 Dyer, A. G., Boyd-Gerny, S., McLoughlin, S., Rosa, M. G., Simonov, V., & Wong, B. B.
413 (2012). Parallel evolution of angiosperm colour signals: common evolutionary
414 pressures linked to hymenopteran vision. *Proceedings of the Royal Society B:*
415 *Biological Sciences, 279*, 3606-3615.
416 Estandia, A., & Sendell-Price, A. T. (2020). Digest: The role of linkage in mimicking "magic
417 traits". In: Wiley Online Library.
418 Fang, B., Kemppainen, P., Momigliano, P., Feng, X., & Merilä, J. (2020). On the causes of
419 geographically heterogeneous parallel evolution in sticklebacks. *Nature Ecology &*
420 *Evolution, 4*, 1105-1115.
421 Feulner, P. G., & Seehausen, O. (2019). Genomic insights into the vulnerability of sympatric
422 whitefish species flocks. *Molecular Ecology, 28*, 615-629.
423 Foll, M., & Gaggiotti, O. (2008). A genome-scan method to identify selected loci appropriate
424 for both dominant and codominant markers: a Bayesian perspective. *Genetics, 180*,
425 977-993.
426 Foster, B. J., McCulloch, G. A., Vogel, M. F. S., Ingram, T., Waters J. M. (2021)
427 Anthropogenic evolution in an insect wing polymorphism following deforestation.
428 *Biology Letters*, 17, 20210069.
429 Friedman, J., Twyford, A. D., Willis, J. H., & Blackman, B. K. (2015). The extent and
430 genetic basis of phenotypic divergence in life history traits in Mimulus guttatus.
431 *Molecular Ecology, 24*, 111-122.
432 Gavrilets, S. (2004). *Fitness landscapes and the origin of species*: Princeton University Press.
433 Gonzalez, D. R., Aramendia, A. C., & Davison, A. (2019). Recombination within the *Cepaea*
434 *nemoralis* supergene is confounded by incomplete penetrance and epistasis. *Heredity,*
435 *123*, 153-161.

436 Gould, B. A., & Stinchcombe, J. R. (2017). Population genomic scans suggest novel genes
437 underlie convergent flowering time evolution in the introduced range of *Arabidopsis*
438 *thaliana*. *Molecular Ecology, 26*, 92-106.
439 Guerra, P. A. (2011). Evaluating the life-history trade-off between dispersal capability and
440 reproduction in wing dimorphic insects: a meta-analysis. *Biological Reviews, 86*, 813-
441 835.
442 Guo, B., Lu, D., Liao, W. B., & Merilä, J. (2016). Genomewide scan for adaptive
443 differentiation along altitudinal gradient in the Andrew's toad *Bufo andrewsi*.
444 *Molecular Ecology, 25*, 3884-3900.
445 Haas, B. J., Delcher, A. L., Mount, S. M., Wortman, J. R., Smith Jr, R. K., Hannick, L. I., . . .
446 Town, C. D. (2003). Improving the *Arabidopsis* genome annotation using maximal
447 transcript alignment assemblies. *Nucleic Acids Research, 31*, 5654-5666.
448 Haas, B. J., Salzberg, S. L., Zhu, W., Pertea, M., Allen, J. E., Orvis, J., . . . Wortman, J. R.
449 (2008). Automated eukaryotic gene structure annotation using EVidenceModeler and
450 the Program to Assemble Spliced Alignments. *Genome Biology, 9*, R7.
451 Haenel, Q., Roesti, M., Moser, D., MacColl, A. D., & Berner, D. (2019). Predictable
452 genome-wide sorting of standing genetic variation during parallel adaptation to basic
453 versus acidic environments in stickleback fish. *Evolution Letters, 3*, 28-42.
454 Härer, A., Bolnick, D. I., & Rennison, D. J. (2021). The genomic signature of ecological
455 divergence along the benthic-limnetic axis in allopatric and sympatric threespine
456 stickleback. *Molecular Ecology, 30*, 451-463.
457 Herman, A., Brandvain, Y., Weagley, J., Jeffery, W. R., Keene, A. C., Kono, T. J., . . .
458 O'Quin, K. (2018). The role of gene flow in rapid and repeated evolution of
459 cave-related traits in Mexican tetra, *Astyanax mexicanus*. *Molecular Ecology, 27*,
460 4397-4416.
461 Hill, J., Enbody, E. D., Pettersson, M. E., Sprehn, C. G., Bekkevold, D., Folkvord, A., . . .
462 Andersson, L. (2019). Recurrent convergent evolution at amino acid residue 261 in
463 fish rhodopsin. *Proceedings of the National Academy of Sciences, 116*, 18473-18478.
464 Hoban, S., Kelley, J. L., Lotterhos, K. E., Antolin, M. F., Bradburd, G., Lowry, D. B., . . .
465 Whitlock, M. C. (2016). Finding the genomic basis of local adaptation: pitfalls,
466 practical solutions, and future directions. *The American Naturalist, 188*, 379-397.
467 Hodkinson, I. D. (2005). Terrestrial insects along elevation gradients: species and community
468 responses to altitude. *Biological Reviews, 80*, 489-513.

469 Hoekstra, H. (2006). Genetics, development and evolution of adaptive pigmentation in
470 vertebrates. *Heredity, 97*, 222-234.
471 Hohenlohe, P. A., Bassham, S., Etter, P. D., Stiffler, N., Johnson, E. A., & Cresko, W. A.
472 (2010). Population genomics of parallel adaptation in threespine stickleback using
473 sequenced RAD tags. *PLoS Genetics, 6*, e1000862.
474 Houot, B., Cazalé-Debat, L., Fraichard, S., Everaerts, C., Saxena, N., Sane, S. P., & Ferveur,
475 J.-F. (2018). Gene regulation and species-specific evolution of free flight odor
476 tracking in *Drosophila*. *Molecular Biology and Evolution, 35*, 3-15.
477 Houot, B., Gigot, V., Robichon, A., & Ferveur, J.-F. (2017). Free flight odor tracking in
478 *Drosophila*: effect of wing chemosensors, sex and pheromonal gene regulation.
479 *Scientific Reports, 7*, 1-10.
480 Hu, Y., Wu, Q., Ma, S., Ma, T., Shan, L., Wang, X., . . . Xiu, Y. (2017). Comparative
481 genomics reveals convergent evolution between the bamboo-eating giant and red
482 pandas. *Proceedings of the National Academy of Sciences, 114*, 1081-1086.
483 Ingham, P. W., & McMahon, A. P. (2001). Hedgehog signaling in animal development:
484 paradigms and principles. *Genes & Development, 15*, 3059-3087.
485 Jackson, J. M., Pimsler, M. L., Oyen, K. J., Strange, J. P., Dillon, M. E., & Lozier, J. D.
486 (2020). Local adaptation across a complex bioclimatic landscape in two montane
487 bumble bee species. *Molecular Ecology, 29*, 920-939.
488 Jones, F. C., Grabherr, M. G., Chan, Y. F., Russell, P., Mauceli, E., Johnson, J., . . . White, S.
489 (2012). The genomic basis of adaptive evolution in threespine sticklebacks. *Nature,*
490 *484*, 55-61.
491 Juan, C., Guzik, M. T., Jaume, D., & Cooper, S. J. (2010). Evolution in caves: Darwin's
492 'wrecks of ancient life' in the molecular era. *Molecular Ecology, 19*, 3865-3880.
493 Kopp, A. (2012). Dmrt genes in the development and evolution of sexual dimorphism.
494 *Trends in Genetics, 28*, 175-184.
495 Kroos, G. C., Waters, J. M., & McCulloch, G. A. (2021). Does assortative mating contribute
496 to reproductive isolation among sympatric ecotypes of the wing-dimorphic stonefly
497 *Zelandoperla fenestrata* (Plecoptera: Gripopterygidae)? *Austral Entomology*.
498 Lai, Y.-T., Yeung, C. K., Omland, K. E., Pang, E.-L., Hao, Y., Liao, B.-Y., . . . Hung, C.-M.
499 (2019). Standing genetic variation as the predominant source for adaptation of a
500 songbird. *Proceedings of the National Academy of Sciences, 116*, 2152-2157.

Leihy, R. I., & Chown, S. L. (2020). Wind plays a major but not exclusive role in the prevalence of insect flight loss on remote islands. *Proceedings of the Royal Society B: Biological Sciences, 287*, 20202121.

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics, 25*, 1754-1760.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., . . . Durbin, R. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics, 25*, 2078-2079.

Love, J., Palmer, J., Stajich, J., Esser, T., Kastman, E., Bogema, D., & Winter, D. (2019). nextgenusfs/funannotate: funannotate v1.7.2 (Version 1.7.2). Zenodo. Retrieved from doi:http://doi.org/10.5281/zenodo.3594559

Lowry, D. B., Hoban, S., Kelley, J. L., Lotterhos, K. E., Reed, L. K., Antolin, M. F., & Storfer, A. (2017). Breaking RAD: An evaluation of the utility of restriction site-associated DNA sequencing for genome scans of adaptation. *Molecular Ecology Resources, 17*, 142-152.

Magalhaes, I. S., Whiting, J. R., D'Agostino, D., Hohenlohe, P. A., Mahmud, M., Bell, M. A., . . . MacColl, A. D. C. (2021). Intercontinental genomic parallelism in multiple three-spined stickleback adaptive radiations. *Nature Ecology & Evolution, 5*, 251-261.

Matthey-Doret, R., & Whitlock, M. C. (2019). Background selection and FST: Consequences for detecting local adaptation. *Molecular Ecology, 28*, 3902-3914.

Matz, M. V. (2018). Fantastic beasts and how to sequence them: ecological genomics for obscure model organisms. *Trends in Genetics, 34*, 121-132.

McCulloch, G. A., Foster, B. J., Dutoit, L., Harrop, T. W. R., Guhlin, J., Dearden, P. K., & Waters, J. M. (2021). Genomics reveals widespread ecological speciation in flightless insects. *Systematic Biology, 70,* 863-876.

McCulloch, G. A., Foster, B. J., Dutoit, L., Ingram, T., Hay, E., Veale, A. J., . . . Waters, J. M. (2019a). Ecological gradients drive insect wing loss and speciation: the role of the alpine treeline. *Molecular Ecology, 28*, 3141-3150.

McCulloch, G. A., Foster, B. J., Ingram, T., & Waters, J. M. (2019b). Insect wing loss is tightly linked to the treeline: evidence from a diverse stonefly assemblage. *Ecography, 42*, 811-813.

[dataset] McCulloch, G. A., Guhlin, J., Dutoit, L., Harrop, T. W. R., Dearden, P. K., & Waters, J. M. (2021). Raw data for "Genomic signatures of parallel alpine adaptation in recently-evolved flightless insects". Datadryad, DOI: https://doi.org/10.5061/dryad.xwdbrv1f0

McCulloch, G. A., Oliphant, A., Dearden, P. K., Veale, A. J., Ellen, C. W., & Waters, J. M. (2019c). Comparative transcriptomic analysis of a wing-dimorphic stonefly reveals candidate wing loss genes. *EvoDevo, 10*, 21.

McCulloch, G. A., Wallis, G. P., & Waters, J. M. (2009). Do insects lose flight before they lose their wings? Population genetic structure in subalpine stoneflies. *Molecular Ecology, 18*, 4073-4087.

McCulloch, G. A., & Waters, J. M. (2018a). Does wing reduction influence the relationship between altitude and insect body size? A case study using New Zealand's diverse stonefly fauna. *Ecology and Evolution, 8*, 953-960.

McCulloch, G. A., & Waters, J. M. (2018b). Testing for seasonality in alpine streams: How does altitude affect freshwater insect life cycles? *Freshwater Biology, 63*, 483-491.

McLellan, I. D. (1999). A revision of *Zelandoperla* Tillyard (Plecoptera: Gripopterygidae: Zelandoperlinae). *New Zealand Journal of Zoology, 26*, 199-219.

Montero-Mendieta, S., Tan, K., Christmas, M. J., Olsson, A., Vilà, C., Wallberg, A., & Webster, M. T. (2019). The genomic basis of adaptation to high-altitude habitats in the eastern honey bee (*Apis cerana*). *Molecular Ecology, 28*, 746-760.

Nagy, L., & Grabherr, G. (2009). *The biology of alpine habitats*: Oxford University Press.

Neupert, S., McCulloch, G. A., Foster, B. J., Waters, J. M., & Szyszka, P. (2020). Adaptive evolution of olfactory degeneration in recently flightless insects. *bioRxiv*, 2020.2004.2010.035311.

Nosil, P., Villoutreix, R., de Carvalho, C. F., Farkas, T. E., Soria-Carrasco, V., Feder, J. L., . . . Gompert, Z. (2018). Natural selection and the predictability of evolution in *Timema* stick insects. *Science, 359*, 765-770.

Ortiz-Barrientos, D., Engelstädter, J., & Rieseberg, L. H. (2016). Recombination rate evolution and the origin of species. *Trends in Ecology & Evolution, 31*, 226-236.

Pan, S., Lin, Y., Liu, Q., Duan, J., Lin, Z., Wang, Y., . . . Shui, G. (2019). Convergent genomic signatures of flight loss in birds suggest a switch of main fuel. *Nature Communications, 10*, 2756.

Peichel, C. L., & Marques, D. A. (2017). The genetic and molecular architecture of phenotypic diversity in sticklebacks. *Philosophical Transactions of the Royal Society B: Biological Sciences, 372*, 20150486.

Price, D. C., Egizi, A., & Fonseca, D. M. (2015). The ubiquity and ancestry of insect doublesex. *Scientific Reports, 5*, 13068.

Privé, F., Luu, K., Vilhjálmsson, B. J., & Blum, M. G. B. (2020). Performing highly efficient genome scans for local adaptation with R package pcadapt Version 4. *Molecular Biology and Evolution, 37*, 2153-2154.

R Core Team. (2019). R: A language and environment for statistical computing. Vienna, Austria.: R Foundation for Statistical Computing.

Rochette, N. C., Rivera-Colón, A. G., & Catchen, J. M. (2019). Stacks 2: Analytical methods for paired-end sequencing improve RADseq-based population genomics. *Molecular Ecology, 28*, 4737-4754.

Roda, F., Walter, G. M., Nipper, R., & Ortiz-Barrientos, D. (2017). Genomic clustering of adaptive loci during parallel evolution of an Australian wildflower. *Molecular Ecology, 26*, 3687-3699.

Roff, D. A. (1990). The evolution of flightlessness in insects. *Ecological Monographs, 60*, 389-421.

Rundle, H. D., & Nosil, P. (2005). Ecological speciation. *Ecology Letters, 8*, 336-352.

Sackton, T. B., & Clark, N. (2019). Convergent evolution in the genomics era: new insights and directions. *Philosophical Transactions of the Royal Society B: Biological Sciences, 374*, 20190102.

Salis, P., Lorin, T., Laudet, V., & Frédérich, B. (2019). Magic traits in magic fish: understanding color pattern evolution using reef fish. *Trends in Genetics, 35*, 265-278.

Salisbury, S. J., McCracken, G. R., Perry, R., Keefe, D., Layton, K. K. S., Kess, T., . . . Ruzzante, D. E. (2020). Limited genetic parallelism underlies recent, repeated incipient speciation in geographically proximate populations of an Arctic fish (*Salvelinus alpinus*). *Molecular Ecology, 29*, 4280-4294.

Servedio, M. R., & Bürger, R. (2020). The effectiveness of pseudomagic traits in promoting divergence and enhancing local adaptation. *Evolution, 74*, 2438-2450.

Servedio, M. R., Van Doorn, G. S., Kopp, M., Frame, A. M., & Nosil, P. (2011). Magic traits in speciation: 'magic' but not rare? *Trends in Ecology & Evolution, 26*, 389-397.

Somme, L. (1989). Adaptations of terrestrial arthropods to the alpine environment. *Biological Reviews, 64*, 367-407.

Soria-Carrasco, V., Gompert, Z., Comeault, A. A., Farkas, T. E., Parchman, T. L., Johnston, J. S., . . . Schwander, T. (2014). Stick insect genomes reveal natural selection's role in parallel speciation. *Science, 344*, 738-742.

601 Storey, J., Bass, A., Dabney, A., & Robinson, D. (2020). qvalue: Q-value estimation for false
602 discovery rate control. R package version 2.22.0. Retrieved from
603 http://github.com/jdstorey/qvalue.
604 Suzuki, T., Suzuki, N., & Tojo, K. (2019). Parallel evolution of an alpine type ecomorph in a
605 scorpionfly: Independent adaptation to high-altitude environments in multiple
606 mountain locations. *Molecular Ecology, 28*, 3225-3240.
607 Terekhanova, N. V., Logacheva, M. D., Penin, A. A., Neretina, T. V., Barmintseva, A. E.,
608 Bazykin, G. A., . . . Mugue, N. S. (2014). Fast evolution from precast bricks:
609 genomics of young freshwater populations of threespine stickleback *Gasterosteus*
610 *aculeatus*. *PLoS Genetics, 10*, e1004696.
611 Todesco, M., Owens, G. L., Bercovich, N., Légaré, J.-S., Soudi, S., Burge, D. O., . . .
612 Imerovski, I. (2020). Massive haplotypes underlie ecotypic differentiation in
613 sunflowers. *Nature, 584*, 602-607.
614 Van Belleghem, S. M., Vangestel, C., De Wolf, K., De Corte, Z., Möst, M., Rastas, P., . . .
615 Hendrickx, F. (2018). Evolution at two time frames: Polymorphisms from an ancient
616 singular divergence event fuel contemporary parallel evolution. *PLoS Genetics, 14*,
617 e1007796.
618 Veale, A. J., & Russello, M. A. (2017). Genomic changes associated with reproductive and
619 migratory ecotypes in sockeye salmon (*Oncorhynchus nerka*). *Genome Biology and*
620 *Evolution, 9*, 2921-2939.
621 Villoutreix, R., de Carvalho, C. F., Soria-Carrasco, V., Lindtke, D., De-la-Mora, M.,
622 Muschick, M., . . . Nosil, P. (2020). Large-scale mutation in the evolution of a gene
623 complex for cryptic coloration. *Science, 369*, 460-466.
624 Waters, J. M., Emerson, B. C., Arribas, P., & McCulloch, G. A. (2020). Dispersal reduction:
625 causes, genomic mechanisms, and evolutionary consequences. *Trends in Ecology &*
626 *Evolution, 35*, 512-522.
627 Waters, J. M., & McCulloch, G. A. (2021). Reinventing the wheel? Reassessing the roles of
628 gene flow, sorting and convergence in repeated evolution. *Molecular Ecology, 17,*
629 4162-4172.
630 Wolf, J. B., & Ellegren, H. (2017). Making sense of genomic islands of differentiation in
631 light of speciation. *Nature Reviews Genetics, 18*, 87.
632 Yeaman, S., Aeschbacher, S., & Bürger, R. (2016). The evolution of genomic islands by
633 increased establishment probability of linked alleles. *Molecular Ecology, 25*, 2542-
634 2558.

Yeaman, S., & Whitlock, M. C. (2011). The genetic architecture of adaptation under migration–selection balance. *Evolution: International Journal of Organic Evolution, 65*, 1897-1911.

Data accessibility

The annotated *Zelandoperla fenestrata* genome is hosted by the national infrastructure platform Genomics Aotearoa, New Zealand (https://www.genomics-aotearoa.org.nz/data). All GBS data used in this study can be found (demultiplexed by sample) in the NCBI Sequence Read Archive PRJNA530622.

Author contributions

J.W. and G.M. conceptualised the study. G.M., J.G., L.D., and T.W.R.H. analyzed the data. G.M. and J.W. drafted the manuscript. All authors contributed to the editing of the manuscript, and gave final approval for publication.

Tables

Table 1. Regions containing outlier SNPs associated with alpine-lowland ecotype differentiation in both Six Mile and Lug Creek *Zelandoperla fenestrata* populations. Genes within 20kb of outliers are listed. Genes in bold have identified functions in insect development that make them strong candidates for roles in the adaptive differentiation between alpine (wing-reduced) versus lowland (full-winged) ecotypes. BS = *BayeScan*, PCA = *pcadapt*. Putative functions of the annotated genes are from http://www.uniprot.org/.

Contig	Position of outliers SNP/s on contig		Annotated genes	Detection method
	Lug	Six Mile		
c192	35394	35394	ppk26	BS,PCA
c323	388712, 388722	373858		BS,PCA
c662	186591, 186595	168784, 186591	RpII15, **dsh**[1,3]	BS
c790	73298, 73327	73298, 73316		PCA
c986	212711, 212719	212711, 212719		BS,PCA
c1774	274485	274536	**abd-A**[2]	PCA
c1781	153552	153543		BS,PCA
c1888	1636037	1651644, 1651654		BS,PCA
c2119	87855	87855, 87924	Nadsyn, **ovo**[3]	BS,PCA
c2925	72997, 83453, 83454, 83488, 83547	83547		PCA
c3220	91435, 91488	91475		BS
c3480	347262	347264		PCA
c4272	9059	9059	Cad96Ca	PCA
c5096	304752	304752	**Mrp4**[1,5]	PCA
c5096	407313	407404	NT1	PCA

c5868	135978	135988		PCA
c6423	53262	53299	**hh**[1,2]	BS,PCA
c17370	181960	179152, 179176	**Desat1**[4,6], Rab14	PCA
s2188	551292, 551307	551292		BS,PCA

[1] wing development, [2] sexual development, [3] fecundity, [4] sensory perception, [5] adult lifespan, [6] mating behavior

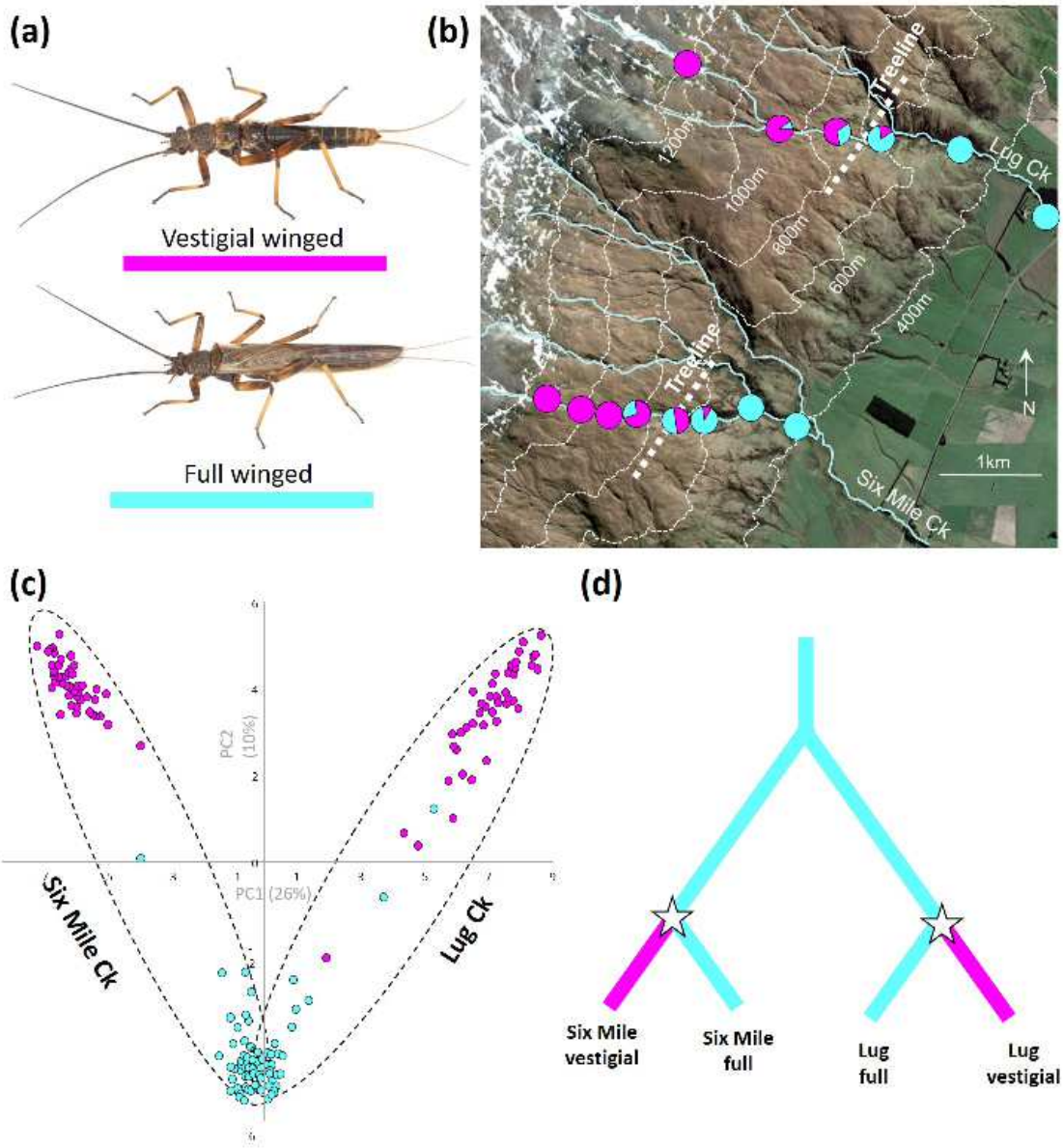

Figure 1.

Repeated evolution of wing-reduced alpine ecotypes in neighbouring Six Mile and Lug Creek stream populations in southern New Zealand. (a) Vestigial-winged and full-winged *Zelandoperla fenestrata* ecotypes. (b) Transect sampling from the Rock and Pillar Range, showing parallel elevational clines in ecotype frequencies (modified from McCulloch et al., 2019a). (c) Principal component analysis illustrating genetic differentiation between vestigial-winged and full-winged *Z. fenestrata* ecotypes within Six Mile Creek (left) and Lug Creek (right) (modified from McCulloch et al., 2019a). (d) Coalescent analysis demonstrating independent ecotype divergence (white stars) in the Six Mile versus Lug Creek *Z. fenestrata* populations (modified from McCulloch et al., 2021).

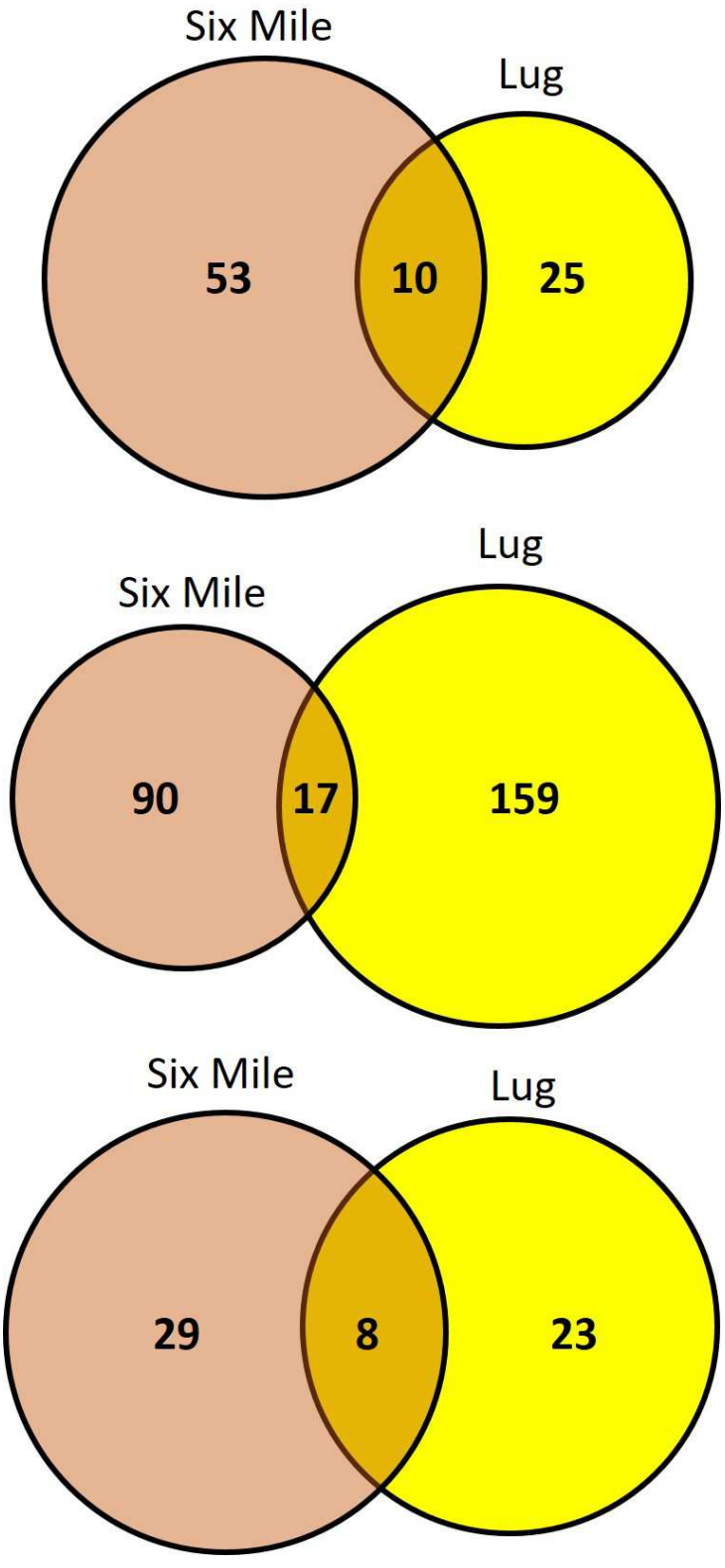

Figure 2.

Shared outlier regions across Six Mile and Lug Creek *Zelandoperla fenestrata* populations. Venn diagrams indicate the number of regions containing outlier SNPs in Six Mile Creek (brown) versus Lug Creek (yellow) populations (and across both streams), as detected using

different analytical approaches. (a) Outliers detected by *BayeScan*; (b) outliers detected by *pcadapt*, and (c) outliers detected by both *BayeScan* and *pcadapt.*

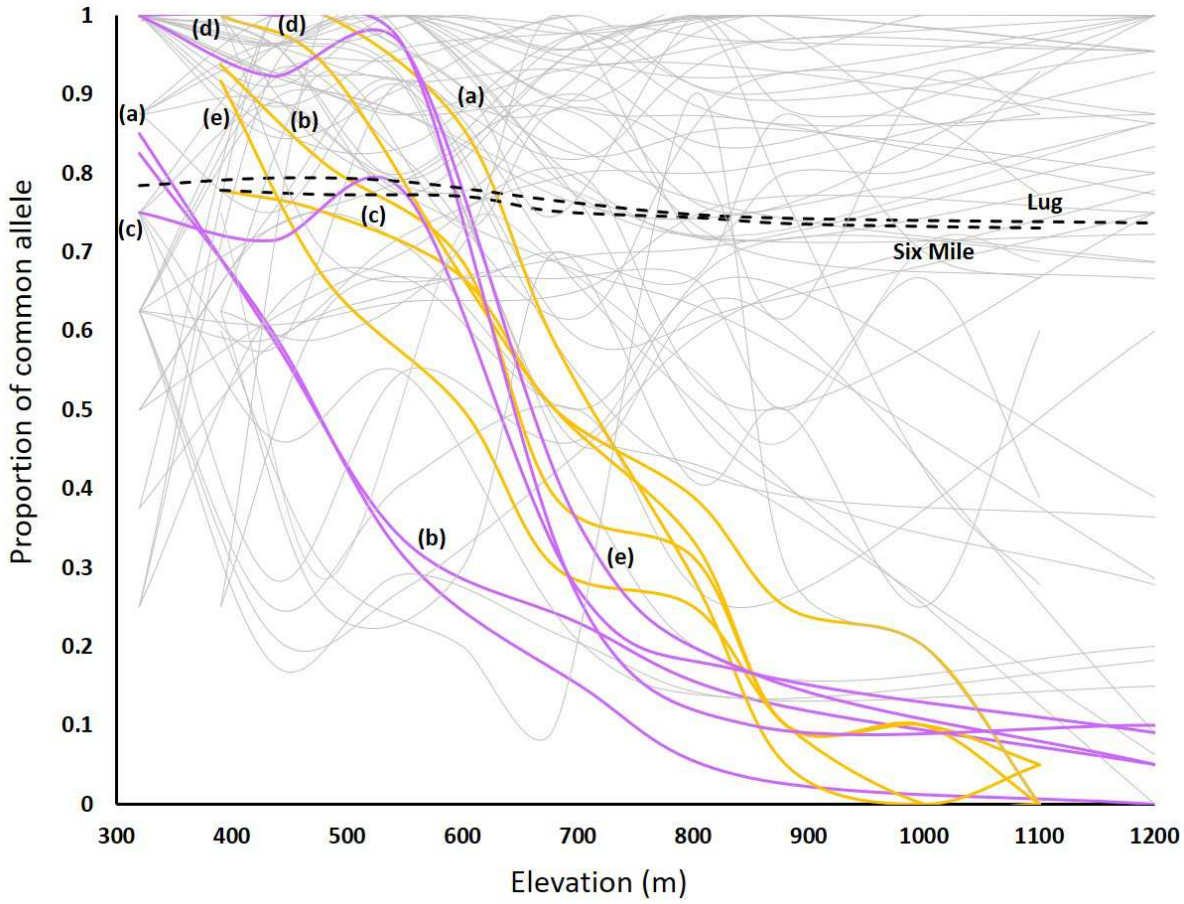

Figure 3.

Parallel elevational clines in the allelic frequencies of outlier SNPs shared across adjacent Six Mile Creek (orange lines) and Lug Creek (purple lines) *Zelandoperla fenestrata* populations. Outlier SNPs are shown from (a) contig 192, (b) contig 790, (c) contig 2925, (d) contig 986, and (e) contig 662 (see Table 1). These outliers are strongly differentiated among ecotypes, and hence broadly track clinal ecotype frequency shifts within each stream (Fig. 1b). The black dashed line indicates the average frequency of the common allele in each stream. Grey lines show 50 randomly selected neutral SNPs.