Summary:

The following is a review of a new version of the manuscript entitled, "Modularity of genes involved in local adaptation to climate despite physical linkage". In general, this version of the manuscript is a nice improvement. In particular, the new introduction and especially the new Figure 1 are far clearer.

This clarity, and the improved understanding of the methods that it provides, bring up a few new technical questions. These are described below under 'major comments'.

Additionally, there are some concepts that remain unclear, in particular, the abstract seems like it could only be understood by a very narrow group of biologists that are familiar with the last decade of literature on pleiotropy. Much of this issue is that important concepts, like modularity, are not explained. Further, terms that have particular meanings like, "genetic architecture," appear to used in a non-traditional way. This is extremely confusing. Most of this issue cannot be resolved by defining terms because these concepts are complex; they merit more detailed explanation than a simple definition. These issues are described below in detail under 'major comments'.

The more I understand, the more I feel confident that this type of analysis, called co-association networks, represents a very cool and useful way to investigate pleiotropy, and further, to define which environmental variables (or combinations of variables) are relevant to natural selection. Still, there are a few things that remain a bit unclear to me, and I hope my comments are helpful to the authors in improving the clarity of the paper to enable a larger collection of biologists to understand and benefit from their work.

Major points:

1. Why do the authors choose 4 clusters in figure 2, rather than 3, or 5, or 6? The authors hint at an answer to this question by stating that the number itself is not important, rather it is the "underlying clustering of the SNPs revealed by co-asssociation networks that is relevant". Still, could that underlying clustering be affected by the number of clusters chosen? It seems important to show that you get the same conclusions about the modular pleiotropy vs. universal pleiotropy debate regardless of how many clusters are chosen for study. Perhaps repeat with different numbers of clusters and show the results do not change? The discussion on lines 538 through 550 also hints at this issue, maybe some of this should be moved up, or there should be more explicit references to the decision about the number of clusters.

2. Definition of modularity. First off, the definition given in table one is circular, i.e. the definition of modularity contains the word "modules". This is not right. The modularity concept is a complex one, and perhaps does not belong in the definitions table but rather in a few sentences, or even a paragraph of the introduction. As written, for a long time I assumed the authors were referring to some specific definition of modules, e.g. genes linked together on a chromosome. But what I think the authors are describing is a more general definition of modules, e.g. genes that all contribute to the same function or process for any number of underlying reasons. In this more general concept of modularity, the underlying reason does not matter, and the modules are defined by the processes to which they contribute, not by some molecular mechanism such as participation in the same gene regulatory network, or reliance on the same transcription factor. This should be described much more thoroughly and earlier.

3. Use of the phrase "genetic architecture". To me, this phrase implies something different from what the authors use it to describe. To me, genetic architecture refers to the location of genes on chromosomes. Therefore a modular genetic architecture is referring to the grouping of genes with similar properties nearby one another on a chromosome. This does not seem to be the only thing the authors mean when they talk about modular genetic architecture. Even in table one, modular genetic architecture says nothing about the locations of genes on chromosomes but says something very generic. I would advise removing the phrase "genetic architecture" from the manuscript. The authors seem to use this phrase to imply something very broad. Perhaps a better word for what the authors mean is "pleiotropic structure", for example, Wagner uses the phrase "pleiotropic structure" in his paper, "the pleiotropic structure of the genotype-phenotype map". The authors of the current manuscript seem to be asking whether the pleiotropic structure is modular, or not.

4. Definitions of complex environments, multivariate environments, intercorrelated environmental variables, aspects of the environment. A key contribution of this manuscript is a better way of defining, understanding and describing the aspects of the environment that influence evolutionary outcomes. As humans, we can measure whatever parameters we can dream up, e.g. temperature, aridity, latitude, but the variables we define by measuring things may not correspond to the primary variables that matter for an organism's fitness. This distinction between environmental variables, and fitness 'aspects', is a really tough one to make. And it is a key part of what this manuscript investigates. These ideas need to be described carefully earlier in the introduction and in plain English, not in fancy statistical terms. In particular, the abstract was really lacking clarity in this area. All it says is that, "intercorrelated environmental variables prevent testing of the predictions…". This statement by itself is so vague! The authors need to think carefully about what issues to tackle in the abstract in more detail, and which issues to omit to save space.

5. Abstract. As mentioned, the abstract was very unclear. I think that in the beginning of the abstract, the authors are trying to describe a problem: linkage among genes under different selective pressures can make natural selection less efficient. This problem should be stated more clearly. The second sentence of the abstract is also unclear because the authors posit a theoretical solution to another, separate problem that they never described. Why would complex environments select for modular genetic architectures? Why would complex environments select for high recombination rates? Why is pleiotropy costly? It is because when genetic changes affect many traits, it becomes more likely that they will have a negative consequence on at least one. This simple fact underlying the "cost of complexity" is never really stated. It should probably be stated in the abstract, so that readers can understand why a modular structure would be expected given complex environments. Also, what is a complex environment? How about something like, "in order to test whether complex environments select for modularity, we must first define what a complex environment is. This is complicated because environmental variables, such as temperature, humidity, and soil composition, are often correlated such that seemingly large numbers of these variables can often be reduced to a single parameter." Please see previous major comment #4 for more thoughts on the abstract.

The abstract should stand alone as a clear description of the paper. In the results section of the abstract, authors use the words, "modules of genes". Does this refer to a set of genes that are physically linked? Modules often refer to genes that perform similar

functions but are not necessarily linked on chromosomes. This was very confusing and it took me all the way through to the results section to really understood what was meant by "modules". I am still only 90% sure I understand.

What could this mean:" We observed limited evidence for environmental pleiotropic effects on distinct aspects of climate?" What is an environmental pleiotropic effect? What is a distinct aspect of climate? Even if this is defined later in a table, it is not OK to use that word here in the abstract without definition.

The abstract should be re-written using simpler concepts and terms.

6. Selection as an all-powerful force. Often times the text implies that natural selection is very powerful and that if something is favored by selection, it should indeed exist in nature. This is not true. For example, in small populations, genetic drift dominates selection. Here is an example of a case where natural selection appears to be described as an all powerful force, lines 553-555: "This was somewhat unexpected from a theoretical perspective, as selection would be expected to disfavour linkage and increase recombination between genes adapting to selection pressures with different spatial patterns of variation [34–36]." In this case, it is not exactly clear what should be expected from a "theoretical perspective". It is entirely possible that selection is weak in this population of trees and does not have the power to rearrange genomes such that genes experiencing similar selection pressures are physically linked. Typically, this kind of strong selection is only expected in microbes with huge populations sizes, and not large organisms with smaller population sizes. In general, the language should be changed to be more careful.

7. Relationships between the text and the figures. The figures are really beautiful and help explain the methods and the results more clearly than the text alone. Often I did not perfectly understand the text, but did understand the figure. In the minor points below, I highlight a number of cases where the overall clarity and impact of the paper could improve substantially if the text takes more time to describe the figures. For example, when describing the opposite associations in the multi vs. the aridity groups in figure 5, point the reader specifically to the green and red dots. Also, the figure legends often seem to describe a specific panel of the figure (e.g. either the left or right side of Figure 1) and yet do not state that this is the case. This leads to erroneous statements about the figures. This should be checked; specific cases are highlighted in the minor points below. Finally, the figures are often miscalled, for example, Figure 1G is repeatedly mentioned but does not exist.

8. Discussion. Often the discussion makes statements that seem bold given the observations. I recommend spending more time connecting the dots: for example, if modularity exists, why does it imply that selection has acted? Lines 612 – 613 describe "drivers of selection", without having made a clear connection between pleiotropy, modularity, and selection pressures.

<u>**Minor points:**</u>
1. The concept of pleiotropy, as well as the second sentence of the introduction, both need more explanation. As previously mentioned, the concept of the "cost of complexity" is a tough one. It is not well summarized by this statement: "Genes that have effects on seemingly unrelated traits may influence the rate of adaptation". What the author means

is: "Pleiotropy is when a gene has effects on multiple traits. Pleiotropy may hinder the rate of adaptation by increasing the likelihood that genetic changes have a deleterious effect on at least one trait."

2. The following sentence starts out clear, but becomes rather unclear. The word "in" does not seem appropriate here. Is this an error? "Because of the conceptual issues described above, certain aspects of genetic architecture have not been well characterized in adaptation to multivariate environments"

3. In the first part of the following sentence, it seems like the authors define modularity as a functional module, but in the second part, it seems like they define modularity as a physical module. Which is it? This issue should be resolved by responding to major comment #2 abut defining modularity. Here is the confusing sentence: "particularly modularity (i.e., when mutations affect traits that are part of the same functional complex, Table 1) and recombination rates among genes in different modules (i.e., physical proximity in the genome)."

4. The phrase on lines 73 and 74 is a bit awkward "aspects of fitness". Is it typical to refer to fitness as being comprised of multiple aspects? This will be resolved when the authors respond to major comment #4, above.

5. The figure legend for figure 1 does not explain what 1a and 1b represent. Are there 4 genes (i.e. 1 through 4) with two SNPs each (i.e. a and b)? If I have this right, then the following sentence in the figure legend is confusing because on the right side the aridity module consists of 4 genes, "For example, the aridity module is a group of SNPs within 2 genes adapting to aridity, and shows associations with both temperature and climate-moisture deficit." Perhaps specify that this refers to the left column?

6. Also, the following sentence in the figure 1 legend is confusing for two reasons: (1) There is a typo (i.e. "are have"), and (2) it is unclear is this is true of both the left and the right examples. "In this example all SNPs within a module are have the same associations with multiple environmental variables."

7. This sentence on lines 210 - 212 is not clear: "Because SNPs in the 4 groups are more dissimilar to SNPs in other groups than the criteria we use to calculate modules, they would not be connected by edges in a co-association network." I do not understand what this sentence means. Does this support the modular pleiotropy hypothesis or the universal pleiotropy hypothesis?

8. The figures referred to in line 235 and 239 are wrong. Figure 1G does not exist.

9. Figure 1G is also referred to in the figure legends, though it does not exist.

10. The statement on lines 272 - 273 needs unpacking. "-association networks capture the same information as simple LD-based clustering with the important additional benefit of linking LD clusters to likely environmental drivers of selection." Is this saying that all of the information you gained could have been revealed by another method? Or does this refer to only specific observations?

11. Figure 3 should be better integrated with the text. Point to specific sections of figure 3 while describing the results in text to improve clarity. As of right now I am confused.

Figure 3 shows that the red contigs seem to be in high LD. But the other contigs (yellow and blue) do not look at all like this. Given Figure 3, I do not understand the line on 291-293. Can you tell me what part of figure 3 demonstrates the conclusion on these lines? Also, these lines actually refer to figure 2, not figure 3, and I think this is a typo.

12. The lines 304 to 307 merit more explanation. What does it mean these SNPS would have been missed? Please clarify the meaning of this sentence. "Overall, 80% of the geography SNPs, 75% of the Freezing SNPs, 20% of the Aridity SNPs, and 10% of the Multi SNPs were *not* outliers along the first 10 PC axes and would have been missed by a study based on PC axes." The section on lines 328 - 336 is very helpful in explaining why co-association is different than PCA.

13. The paragraph on line 346 uses the words "expected genome-wide covariance", and I have interpreted this to mean that the expectation is based on whether environmental variables are themselves correlated. Maybe add more information to clarify, perhaps something like, "We can visualize (i) the expected genome-wide covariance (given correlations between environmental variables Fig 1A left panel) using shading of quadrants and (ii) the observed genome-wide covariance using a 95% prediction ellipse (Figure 4).

14. Maybe help the reader see the following pattern by pointing to it very clearly in text. Something like, "Galaxy biplots revealed that SNPs in the Aridity group showed associations with hot/dry versus cold/wet environments, while SNPs in the Multi and Freezing groups showed **the opposite** patterns of associations with hot/wet versus cold/dry environments (Figure 5A; **red vs green points demonstrate the opposite correlations**). In general, more connections between the text and the figures would be very helpful.

15. The results section should specify how the correcting the associations for population structure were performed in Figure 5B.

16. In line 430, it seems premature to call these "adaptation clusters". The analysis on lines 370 through 428 is indeed very intriguing and suggests that these clusters of SNPs adapted to different selection pressures. But calling them adaptation clusters seems like a jump.

17. In lines 578-588, the phrasing is awkward. For example, in the following sentence, the word plastic seems unfair to use: "However, differential expression analyses only identify genes with transcription (i.e., plastic) responses to climate". Plastic and plasticity have specific meanings in the literature, and it seems here those words are adopted for a narrow usage. This can cause a lot of confusion. In general, there is a lot of reliance on definitions in this manuscript, and the authors define terms that have a more general meaning in a narrow way. Instead, can the authors try to describe things more simply? For example, this sentence is so confusing: "We also compared the modularity of plastic responses to climate (as measured by co-expression networks) to the modularity of evolved genetic responses to climate (as measured by co-association networks)." Why not stick to the basics and instead say: "We also compared co-expression networks to co-association networks." I do not see any need to use the word plasticity here.

18. The conclusions in lines 590 – 597 seems very strong, and not necessarily justified by the data. Perhaps tone down the language here to make these suggestions rather

than conclusions. Also, see major comment #2 regarding definition of modularity. It seems in the discussion the authors finally expand on this concept. Maybe this should come earlier.