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IDENTIFICATION OF GENES UNDER DIVERGENT SELECTION IN INTERFERTILE, BUT ECOLOGICALLY DIVERGENT OAKS

Oliver GAILING[1]

Abstract: *In early stages of speciation with gene flow, divergent selection creates genomic regions with elevated levels of differentiation (outlier regions) interspersed by larger genomic regions that are homogenized by gene flow. Ecologically divergent, but interfertile oaks show such a pattern of genomic divergence and thus provide a system to identify outlier regions. The availability of a reference genome in oaks in the near future will allows us to anchor these outlier regions to the genome sequence and to identify genes with putative role in adaptive divergence and reproductive isolation between species. Current advances in outlier screens in oaks with specific emphasis on American red oaks (section Lobatae), and comparative outliers across oak sections are reviewed. In addition, strategies are outlined to test for associations of nucleotide variation in putative outlier loci with adaptive traits that are involved in adaptive species divergence and reproductive isolation.*

Keywords: *Outlier genes, Quercus, ecological speciation, SSRs, genome scans.*

1. Introduction

Natural selection drives speciation [41], [42] and the evolution of barriers to interspecific gene flow [39], [42], [49], [50]. However, little is known about the underlying genes that are involved in adaptive species divergence and reproductive isolation between species [37], [44]. In the face of interspecific gene flow, divergent selection will generate a genomic mosaic of regions that are homogenized by interspecific gene flow punctuated by regions with high interspecific differentiation as result of strong divergent selection (outlier regions) [35], [43], [50]. Divergent selection on outlier loci reduces effective gene flow and recombination around these genes ("divergence hitchhiking") [49], [50], which could result in the accumulation of alleles involved in reproductive isolation [50]. Due to recurrent interspecific gene flow, oaks provide a model to study genomic signatures of divergent selection [21], [43]. Thus, genome scans in the ecologically divergent and interfertile species *Q. robur* and *Q. petraea* revealed a pattern of genomic divergence [43] predicted by the models of early stages of speciation with gene flow and strong divergent selection [49], [50].

[1] Michigan Technological University, 1400 Townsend Drive, 49931 Houghton, MI, United States.

Given the availability of the *Q. robur* genome sequence in the near future (Christophe Plomion, pers.comm., https://w3.pierroton.inra.fr/QuercusPortal/index.php?p=OAK_GENOME_SEQUENCING), genome scans in hybridizing, but ecologically divergent oaks can be used to identify genomic regions with signatures of divergent selection. Genetic linkage maps in the European white oaks [8], [14], [19] and in the North American red oaks (Jeanne Romero-Severson, pers. comm.) are the basis for Quantitative Trait Locus (QTL) mapping to analyze the association of genetic variation in outlier regions with adaptive trait variation. To narrow down the outlier regions to individual genes, they can be anchored to the *Q. robur* genome sequence and underlying candidate genes for association mapping studies can be identified.

Here, I present results on outlier screens in the North American red oaks (section *Lobatae*) and highlight strategies to identify genes under divergent selection across taxonomic groups and to evaluate their association with traits related to adaptive species divergence and reproductive isolation.

2. Genetic and morphological species boundaries in red oak species (section *Lobatae*)

Genetic and morphological species boundaries in the North American red oaks are often ambiguous [4], [24]. For example, very low mean interspecific genetic differentiation was observed at randomly selected nuclear Simple Sequence Repeat (SSR) and genic Expressed Sequence Tag (EST)-SSR markers (e.g. < 5% between *Q. rubra* and *Q. ellipsoidalis*; [30], [47]). Likewise, separation between the related and interfertile species *Q. rubra* and *Q. ellipsoidalis* at 16 leaf morphometric traits was incomplete likely as the result of phenotypic plasticity and incomplete reproductive isolation between species [21]. Independent evidence from genetic assignment analyses at SSR and Amplified Fragment Length Polymorphism (AFLP) markers [24], [30], [36], [48] and chloroplast DNA analyses in *Q. rubra* / *Q. ellipsoidalis* and in *Q. velutina* / *Q. ellipsoidalis* population pairs (Zhang et al., in prep.) illustrated recurrent gene flow among species. Yet despite interspecific gene flow between red oak species, species cluster genetically [30], [36] and remain ecologically distinct [1], [2], [18].

The overall very low interspecific genetic differentiation among hybridizing red oak species at genome-wide AFLPs [24], at most nuclear and EST-SSRs [30], [47] and at chloroplast DNA markers (Zhang et al., in prep.) suggested that most of the genome is homogenized by interspecific gene flow.

3. Initial outlier screens between *Q. rubra* and *Q. ellipsoidalis* with randomly selected markers

Quercus rubra and *Q. ellipsoidalis* are interfertile oak species of section *Lobatae* that show different adaptations to drought as reflected for example in differences in root depth, leaf conductance and tissue elasticity [1].

Outlier screens using 15 randomly selected nuclear and gene-based EST-SSRs revealed a few loci with values of interspecific differentiation above neutral expectations between neighboring stands of *Q. rubra* and *Q. ellipsoidalis* [30].

Thus, two loci, GOT021 and 3A05, were identified as outliers under divergent selection between species in adult and seedling generations [10], [30]. GOT021 is a gene-linked EST-SSR in the 5'UTR region of a histidine kinase 4-like gene (Hk4) that was originally developed in

Q. robur [14], while 3A05 is a nuclear SSR marker originally developed for *Q. rubra* [5] with unknown genomic location and function. Interestingly, GOT021 was fixed on one allele in a *Q. ellipsoidalis* seedling population and showed strongly reduced genetic variation in *Q. ellipsoidalis* adult trees and acorns as compared to *Q. rubra* suggesting purifying selection [10]. Likewise, GOT021 showed signatures of purifying selection in neighboring *Q. rubra* / *Q. ellipsoidalis* stands from another region [29], [47].

Quercus ellipsoidalis grows on dry outwash plains and is characterized by deeper tap roots than *Q. rubra* and by wide-spreading deep lateral roots [1]. Interestingly, Hk4 was found to be a major regulator of root growth and function in *Arabidopsis* [12] and triple mutants (Hk2/Hk3/Hk4) showed growth inhibition for example in leaves, roots and inflorescence meristems [34]. GOT021 was also underlying a QTL for leaf shape variation in a *Q. robur* full-sib family [19].

4. Outlier screens at selected gene-based markers between *Q. rubra* and *Q. ellipsoidalis*

A *CONSTANS-like* gene as outlier under strong divergent selection

Outlier screens using 36 genic EST-SSRs with potential function in abiotic stress tolerance, phenology and growth, that were originally developed in *Q. robur* and *Q. petraea* [14], and eight nuclear SSRs [47] confirmed GOT021 as an outlier and identified four additional gene-based outlier loci (FIR013, FIR039, POR016, GOT040). One of these outliers, FIR013, was identified as under strong divergent selection in all four interspecific population pairs from three geographic regions [29]. The microsatellite was nearly fixed for alternative alleles (138 bp in *Q. ellipsoidalis*, 141bp in *Q. rubra*) in the two species and consequently showed very high interspecific differentiation (F_{ST} = 0.38 – 0.79) in adult and seedling generations [10], [29]. Migrant alleles and heterozygotes (138 bp/141 bp) were rare in each region, but more frequent in the *Q. ellipsoidalis* populations which might indicate asymmetric gene flow between the more frequent *Q. rubra* and the disjunct *Q. ellipsoidalis* populations [29].

FIR013 is a tri-nucleotide SSR that was derived from an EST annotated as a *CONSTANS-like* gene (*COL*) [29], a candidate gene for flowering time [51]. It encodes a poly (E) repeat. The *Q. ellipsoidalis* allele (138 bp) is characterized by the deletion of one glutamine residue [29]. Interestingly, a poly (E) repeat allele in another *COL* gene (*COL2B*) was associated with growth cessation in *Populus tremula* [32]. Also, nucleotide variation in the same *COL* gene that differentiated between *Q. rubra* and *Q. ellipsoidalis* was associated with bud flush timing in *Q. petraea* [3]. *CONSTANS-like* genes are zinc finger transcription factors that play an important role in the photoperiod pathway of floral transition [6] and in growth and development [23], [25]. For example, in *Medicago sativa* a *COL* gene was associated with both height and flowering time [23]. Consequently, *COL* genes may play a role in both adaptive divergence and reproductive isolation between species [29]. Interestingly, *Q. ellipsoidalis* seedlings showed a higher mortality and a significantly later, though overlapping, bud burst than *Q. rubra* seedlings in a common garden trial [18].

Associations of variation in *COL* with fitness traits related to adaptive species divergence and reproductive isolation can be tested in interspecific progenies that segregate for parental trait differences or in hybrid or single-species populations that

show heritable variation for these traits (see below). Since oaks show considerable within-species variation for traits that differentiate between species, also intraspecific crosses were used to map Quantitative Trait Loci (QTL) for interspecific trait differences [38], [43]. For example, in an intraspecific *Q. robur* progeny an outlier locus (QrZAG96) between *Q. robur* and *Q. petraea* [43] was found to underlie a QTL for a leaf trait (petiole ratio) that showed very high differentiation (84%) between both species [38], [43].

So far, outlier screens between *Q. rubra* and *Q. ellipsoidalis* population pairs were performed in the northern range of their sympatric distribution where both species occur on highly contrasting sites (mesic vs. xeric conditions). However, *Q. ellipsoidalis* populations also grow under more mesic conditions at the southern extent of the species' distribution (A. Hipp, pers. comm.). If divergent selection maintains allele frequency differences between species, it is conceivable that interspecific differentiation at outlier loci varies with the steepness of environmental gradients between interspecific population pairs. Thus, species-discriminating genes could be nearly fixed on alternative alleles in highly contrasting environments as observed for *COL* [29], while in regions with less pronounced micro-environmental differences introgression of adaptive alleles between species could be more frequent. Results from a narrow geographic range indicated that the frequency of introgressed alleles at *COL* differed for geographic regions with different edaphic conditions [29]. Thus the interfertile species *Q. rubra* and *Q. ellipsoidalis* could provide a model to study the effect of environmental gradients on introgression of outlier alleles. In regions of more pronounced introgression, the analysis of associations of migrant and local alleles with adaptive traits using admixture mapping approaches might be feasible.

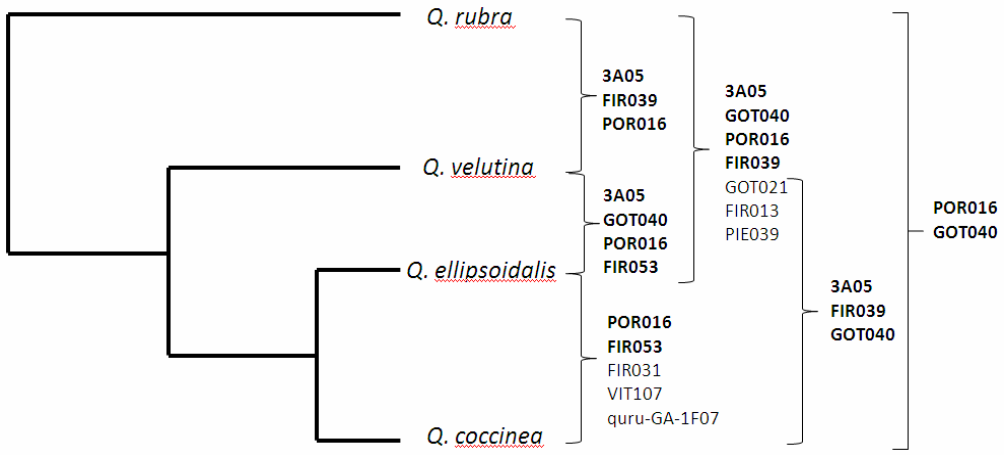

Fig. 1. *Phylogenetic relationships between four red oak species with different adaptations to drought [36]. Outlier loci between species pairs are shown next to brackets. Printed in bold are markers that were identified between two or more species [30], [29], [47], [48]. The major outlier FIR013 (COL) has only been tested between "Q. rubra" and "Q. ellipsoidalis"*

5. Additional outliers with moderate interspecific differentiation

The other outliers between *Q. rubra* and *Q. ellipsoidalis* (GOT021, GOT040, POR016 and FIR039) showed much lower interspecific differentiation than FIR013 and were identified in only one or two of the three geographic regions [29]. Two of these markers, GOT040 and POR016, were found as under divergent and purifying selection in the same geographic region. Both markers are located on the same bin of linkage group 6 (≤ 15 cM apart) of *Q. robur* [14]. GOT040 and POR016 were annotated as 40s ribosomal 16s-like protein and as serine/threonine-protein phosphatase 5-like protein [47], two genes with unknown function in drought tolerance or reproductive isolation. Additionally, FIR039 was identified as under purifying selection in *Q. ellipsoidalis* [29], [47]. FIR039 was derived from an EST that was annotated as a putative histone deacetylase, a gene that affects both flowering time [13] and stress responses [53] in other species. Thus, FIR039 could be a candidate gene for both adaptive divergence and reproductive isolation [47].

6. Comparative outliers in section *Lobatae*

Quercus ellipsoidalis and *Q. velutina* are more closely related than *Q. ellipsoidalis* and *Q. rubra* (Figure 1) and interspecific gene flow between neighboring populations is more frequent [24], [48]. An outlier screen between neighboring populations of the ecologically divergent species *Quercus velutina* and *Q. ellipsoidalis* also identified the two gene-based EST-SSRs GOT040 and POR016 on linkage group 6 as outliers [48].

Genetic variation and number of alleles at both markers were strongly reduced in all five *Q. ellipsoidalis* populations across the Great Lakes region indicative of a recent selective sweep [48]. Also, 3A05 and an additional gene marker, FIR053, were identified as outliers under divergent selection between *Q. velutina* and *Q. ellipsoidalis*. FIR013 has not yet been tested in other red oak species pairs than *Q. rubra* and *Q. ellipsoidalis*.

Additional outliers were identified between allopatric interspecific populations; EST-SSRs FIR031, FIR053, VIT107, POR016 and nuclear SSR quru-GA-1F07 between the sister species *Q. ellipsoidalis* and *Q. coccinea*, EST-SSRs FIR039 and POR016, and nuclear SSR 3A05 between *Q. rubra* and *Q. velutina*, EST-SSR PIE039 between *Q. ellipsoidalis* and *Q. rubra*, 3A05, FIR039 and GOT040 between *Q. velutina* and *Q. coccinea*, and GOT040 and POR016 between *Q. rubra* and *Q. coccinea* [48] (Figure 1).

In total five markers were identified as outliers between more than one species pair. All outlier loci are plotted on a phylogenetic tree of four interfertile members of section *Lobatae* (Figure 1). The largest number of markers and population pairs were analyzed between *Q. rubra* and *Q. ellipsoidalis* and between *Q. velutina* and *Q. ellipsoidalis*. Three of the four outliers detected between neighboring *Q. velutina* and *Q. ellipsoidalis* populations, 3A05, GOT040, POR016, were also identified between neighboring *Q. rubra* and *Q. ellipsoidalis* populations.

Some outliers differentiated derived clades from ancestral species (Figure 1). Thus POR016 was identified as outlier between *Q. rubra* and each of the derived species *Q. velutina*, *Q. ellipsoidalis* and *Q. coccinea*. Likewise, 3A05 and GOT040 were outliers between *Q. velutina* and each of the two sister species *Q. ellipsoidalis* and *Q. coccinea*. The analyses of larger numbers of markers using genome scans based on next generation sequencing will

allow us to reconstruct the evolution of outliers within these four species with different adaptations to drought.

7. Comparative outlier regions across oak sections

Comparative outlier analyses across taxonomic groups can reveal parallel genomic divergence driven by natural selection as shown recently in an animal model [45]. In oaks, the most comprehensive outlier screens have been performed between the European white oak species *Q. robur* and *Q. petraea* (section *Quercus*) revealing a mosaic of genomic regions with low interspecific differentiation interspersed by regions with signatures of divergent selection and a hotspot of interspecific differentiation on linkage group 12 [43]. Both interfertile species co-occur in most European forests, but their distribution is restricted by different soil preferences, and interspecific hybrids occur in the contact zones between species [11]. Thus, *Q. petraea* prefers drier soils, while *Q. robur* is more frequent on nutrient rich soils which are temporally subjected to flooding [9], [28], [52]. One nuclear SSR marker, QrZAG112, has been identified as an outlier between *Q. robur* and *Q. petraea* (section *Quercus*) [22], [43] as well as between the species pair *Q. alnifolia* and *Q. coccifera* from section *Cerris* [33]. Likewise, the *COL* gene was identified as a strong outlier between *Q. rubra* and *Q. ellipsoidalis* [29] and showed elevated levels of interspecific differentiation between the European white oaks *Q. robur* and *Q. pedunculiflora* K. Koch (Curtu et al., in prep.), two closely related species of section *Quercus* with different adaptations to drought [15], [20].

Until now comparative outlier screens in different oak sections were restricted to a few gene markers. Next generation sequencing such as Restriction Site Associated DNA (RAD) sequencing [7] offers the opportunity to analyze genome-wide patterns of interspecific divergence [26], [46]. When sequence reads are anchored to the *Q. robur* whole genome sequence (WGS), genomic regions with signatures of divergent selection can be compared to reveal patterns of parallel genomic divergence.

In addition, comparisons of multiple interfertile taxa within sections (e.g. both within section *Quercus* and *Lobatae*) with different divergence times could reveal the sequential accumulation of barrier loci (loci under divergent selection and/or involved in reproductive isolation between species) in the genome (Figure 1).

8. Association of outlier alleles and adaptive species differences

QTL and association mapping studies could show whether variation at outlier loci or linked genes (e.g. *COL*) is associated with traits that are related to adaptive divergence and reproductive isolation between species (e.g. phenology, growth traits or drought tolerance).

A QTL mapping population has been established for *Q. rubra* and the development of a high-density genetic linkage map is under way (Jeanne Romero-Severson, pers. comm.). Also, range-wide provenance trials for *Q. rubra* are available for future association mapping [27], [40]. Likewise, QTL and association populations and linkage maps are available for the European white oaks (see above, [8], [3]).

QTL mapping can be used to identify chromosomal regions that show significant associations with adaptive traits, and co-locations with putative outlier loci. Due to the rapid decay of linkage disequilibrium in natural populations of outcrossing trees [17] association mapping can be applied to test for associations of nucleotide variation

within individual genes with adaptive traits and climatic / environmental parameters [3], [16]. For example, whole genome outlier screens in combination with association analyses revealed a large number of genomic regions that showed both associations with adaptive trait variations in *Populus trichocarpa* and signatures of recent positive or divergent selection among populations [16]. Likewise, a *Q. petraea* provenance trial was used to assess associations of nucleotide variation with adaptive traits such as bud burst and environmental variables of source populations [3].

Interestingly, a candidate gene for phenology (*COL*) was significantly associated with bud burst in this provenance trial [3], and the same gene was also found as major outlier between *Q. rubra* and *Q. ellipsoidalis* (section *Lobatae*) [29].

Also, admixture mapping in hybrid zones holds promise to identify marker-phenotype associations (QTL) for adaptive species differences [31]. For example, admixture mapping for leaf traits in *Populus tremula* / *P. alba* hybrid zones found significant marker-phenotype associations (QTL) on several linkage groups each explaining moderate amounts of the total phenotypic variation (from 2.3% to 18.2%), and some of the markers were consistently associated with the same leaf traits in more than one hybrid zone [31].

In oaks, genome scans could be applied to identify outlier loci and to assess their association with adaptive species differences in the same hybrid zone using admixture mapping approaches. Outlier loci under divergent selection between species that are also associated with adaptive species differences are candidate genes for adaptive divergence and reproductive isolation between species.

9. Conclusions

Outlier screens in interfertile, but ecologically divergent oak species can identify genomic regions under divergent selection. These regions can be anchored to the reference genome sequence of *Q. robur* to pinpoint underlying genes with putative role in adaptive divergence and reproductive isolation between species. Association of variation in these genes with adaptive traits can be analyzed in QTL or association populations, or using admixture mapping in hybrid zones. Next generation sequencing derived markers facilitate genome scans for outlier genes and association analyses.

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