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ORIGINAL ARTICLE

Assessing model fit in phylogeographical investigations: an example from the North American sandbar willow *Salix melanopsis*

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ABSTRACT

Aim Coalescent models enable the direct estimation of parameters with clear biological relevance (i.e. divergence time, migration rate and rate of expansion), but they have typically been applied to phylogeographical research without a priori assessment of their fit to the empirical system. Here we explore the extent to which phylogeographical inference can be misled by evaluating the fit of several population genetic models to empirical data collected from the sandbar willow, *Salix melanopsis*.

Location The Pacific Northwest mesic forest of North America.

Methods We collected sequence data from five loci in 145 individuals. We assessed model fit in: (1) models delimiting previously proposed races within *S. melanopsis*; (2) historical biogeographical models, each describing the timing and pattern of diversification; and (3) coalescent models that correspond to those implemented in popular software packages such as IMa, LAMARC, and MIGRATE-N.

Results We found little evidence for previous hypotheses of cryptic races delimited by habitat type (mesic, lowland or subalpine); rather, our results suggested that these variants originated from the same source population. Historical biogeographical models demonstrate that *S. melanopsis* has recently expanded from a single refugial population, probably located in the northern Rocky Mountains. An analysis using approximate Bayesian computation indicated that the single population expansion model implemented in LAMARC is a better fit to the data than multi-population models incorporating migration and/or divergence as implemented in MIGRATE-N and IMa, suggesting that the parameters estimated from the latter are potentially misleading for this system.

Main conclusions Our research highlights the importance of assessing model fit in addition to estimating parameters to understand evolutionary processes. Taken together, they allow us to infer the historical demography of *S. melanopsis* in a manner that is not biased by previous work in the system.

Keywords

Approximate Bayesian computation, model selection, Pacific Northwest, phylogeographical method, phylogeography, *Salix*, species delimitation.

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INTRODUCTION

Phylogeographical investigations are increasingly conducted using a model-based framework because the use of mathematical models such as species trees and the coalescent enable the direct estimation of parameters with clear biological relevance (e.g. phylogeny, divergence time, migration rates and rate of expansion). However, it is common for models to be applied to particular phylogeographical systems without a priori assessment of their statistical fit to the system, despite the fact that parameter estimates can vary, at times substantially, under different models (e.g. Koopman & Carstens, 2010). Here we explore the issue of phylogeographical model fit by quantifying the fit of models to empirical

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data collected from the sandbar willow, *Salix melanopsis*. We assess model fit in the following three instances: (1) models that seek to identify the limits of cryptic evolutionary lineages or races; (2) historical biogeographical models, each describing the timing and pattern of diversification; and (3) models that correspond to those implemented in popular software packages and that are intended to estimate relevant biological parameters such as divergence time, migration rates and population expansion using a particular coalescent model. Our data are representative of many phylogeographical studies (sequence data from five loci in 145 individuals) and, taken together, these models and our data span the range of aims from identifying cryptic lineages to estimating divergence times common to model-based phylogeographical research.

Biogeographical hypotheses pertaining to the Pacific Northwest mesic forests

The temperate rain forests of the Pacific Northwest of North America are characterized by a cool and wet maritime climate that occurs at high elevations along the Pacific coast and inland in the northern Rocky Mountains. Major geological events such as mountain orogeny, xerification and glaciation have influenced the evolution of the more than 150 eukaryote species that inhabit these forests (Brunsfeld *et al.*, 2001). While the physical history and previous work in this region have been recently reviewed (Shafer *et al.*, 2010), a basic outline will help to clarify the historical models we will evaluate with our data. Mesic forests in this region date to the formation of the northern Rockies during the Eocene (Graham, 1999) and began to form on the nascent Cascade Range and coastal ranges during the Pliocene. By the beginning of the Pleistocene, the rising Cascades had created a rain shadow that dried out the Columbia Basin, effectively separating the coastal and the inland rain forests (Graham, 1999). Glaciers advanced and retreated throughout the Pleistocene (Pielou, 1991), forcing mesic forest endemics into refugia located in southern river canyons in both the northern Rocky Mountains and the Cascade Range (Barnosky *et al.*, 1987). The geological history of the region inspired Brunsfeld *et al.* (2001) to propose general hypotheses that could be tested with genetic data. In general, these hypotheses (Fig. 1) seek to explain the coastal/inland disjunct distribution as either a function of an ancient (pre-Pleistocene) vicariance (with subsequent refugial persistence during glaciation in both the Cascade Range and northern Rocky Mountains) or of recent dispersal from one region after the Pleistocene. Refugia were originally conceived as single populations, but evidence of deep phylogeographical structure in some species led Brunsfeld & Sullivan (2005) to propose that some taxa persisted in compartmentalized refugia in multiple river canyons. The data described below were collected in order to evaluate these hypotheses. However, in order to evaluate these models using genetic data, it is critical to first assess the limits of evolutionary lineages and to identify

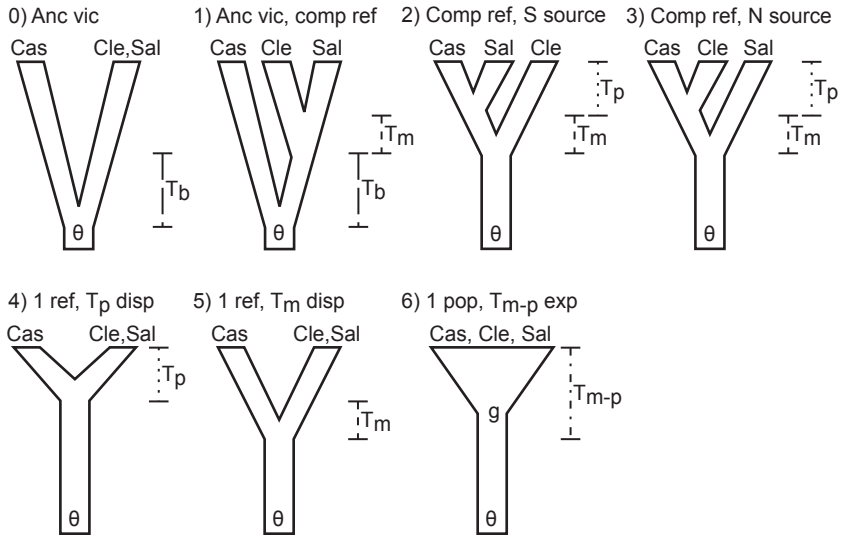

Figure 1 Possible models of the range expansion of *Salix melanopsis* in the Pacific Northwest used in the approximate Bayesian computation analysis. The models are: (0) ancient vicariance between the Cascade Range and northern Rocky Mountains; (1) ancient vicariance followed by differentiation into compartmentalized Rocky Mountain refugia in the Clearwater and Salmon River regions; (2) compartmentalized refugia in the Rockies followed by colonization of the Cascades from the southern Salmon River region; (3) compartmentalized refugia followed by colonization of the Cascades from the northern Clearwater region; (4) a single refugium, post-Pleistocene dispersal to the Cascades; (5) a single refugium, mid-Pleistocene dispersal to the Cascades; (6) a single population with expansion beginning in the mid-Pleistocene and continuing to the present. Cas = Cascades; Cle = Clearwater; Sal = Salmon River regions. Model parameters: $\theta = 4N_e\mu$; γ = intrinsic growth rate; T_b = divergence time before the Pleistocene; T_m = divergence time mid-Pleistocene; T_p = divergence time post-Pleistocene; T_{m-p} = timing of expansion from the mid-Pleistocene to the present.

specific population processes that need to be parameterized in this system.

Like other members of *Salix* sect. *Longifolia*, *S. melanopsis* Nutt. occupies the banks of streams and rivers. Members of the section are diploid (Brunsfeld *et al.*, 1992), morphologically distinct from other *Salix* species (Argus, 1997), and their chloroplast genomes are divergent from their congeners (Brunsfeld *et al.*, 2001). *Salix* species are dioecious; their catkins open in the early spring and pollen is spread by wind and insects (Peeters & Totland, 1999), although the latter are probably the more effective (Sacchi & Price, 1988). Despite the distinctness of *Salix* sect. *Longifolia* from other *Salix* taxa, relationships within this section are not clear, perhaps due to the hybrid origin of species such as *Salix fluviatilis* (Argus, 1997). Initial taxonomic work described between one and sixteen species (Bebb, 1891; Schneider, 1919), and the most recent monographic treatment identified seven (Argus, 1997). Despite these taxonomic difficulties, *S. melanopsis* is distinct and not similar to other members of the section (Argus, 1997), making this a suitable species for phylogeographical investigation. Moreover, the genetic data used to confirm the distinctness of *Salix* sect. *Longifolia* from other *Salix* taxa suggest that there is cryptic diversity (i.e. independent evolutionary lineages) within *S. melanopsis* (Brunsfeld *et al.*, 1992, 2007). Brunsfeld *et al.* (1992) proposed that *S. melanopsis* contained three races (lowland, mesic and subalpine) based largely on habitat type and morphology, and indicated that multiple races could be identified within a single locality. We were therefore hesitant to analyse all the morphologically identified *S. melanopsis* individuals without first assessing this cryptic diversity.

We explore this question of species boundaries using herbarium samples collected during previous studies (Brunsfeld *et al.*, 1992; Carstens *et al.*, 2005b; Brunsfeld *et al.*, 2007) and follow both a discovery approach using a genetic clustering method (Pritchard *et al.*, 2000) and multiple lineage validation approaches that use species tree methods (Yang & Rannala, 2010; Ence & Carstens, 2011) in order to evaluate any cryptic diversity. As most model-based phylogeographical methods assume some partitioning of samples into groups, our goal with the species delimitation analysis was to ascertain whether the races described by Brunsfeld *et al.* (1992) represent independent biological lineages and to help us to understand how the samples should be partitioned prior to the historical model analysis. We also explore a major assumption of the species delimitation methods, namely that the shared polymorphism results from incomplete lineage sorting. Because the accuracy of methods such as SpedeSTEM can be decreased if some of the shared polymorphism results from gene flow (Ence & Carstens, 2011), we evaluate the relative fit of two coalescent models that include gene flow – implemented in the software packages Migrate-n (Beerli & Felsenstein, 2001) and IMa (Hey & Nielsen, 2007) – to one that does not (lamarc; Kuhner, 2006).

MATERIALS AND METHODS

Sampling, DNA extraction, PCR and sequencing

Leaf tissue from 145 individuals of *S. melanopsis* was either collected in the field and dried on silica or borrowed from the Stillinger Herbarium at the University of Idaho. The samples represented populations from five river drainages in the Cascade Range and from 12 river drainages in the northern Rocky Mountains (see Table S1 in Supporting Information). One individual of *Salix alba* from Michigan was also included to act as an outgroup where appropriate. Whole genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions and quantified using a spectrophotometer.

Sequence data were collected from five independent loci. One of these was the chloroplast locus *mat*K, where 1159 bp were collected from samples not included in Brunsfeld *et al.* (2007), following the protocol given in that paper. Sequence data from four nuclear loci were collected using primers developed from the *Populus trichocarpa* genome (Tuskan *et al.*, 2006) using the web-based genome browser provided by Phytozome (available at http://www.phytozome.net/poplar). *Populus* is an ancient polyploid, so care was taken to choose regions present as a single copy in *Populus* to avoid issues of paralogy. We initially designed 18 primer pairs following the EPIC approach described by Palumbi & Baker (1994), and a screening panel of three *S. melanopsis* individuals was used to evaluate these markers using the following approach: (1) polymerase chain reaction (PCR) was attempted using a gradient of annealing temperatures and $MgCl_2$ concentrations using Phusion high-fidelity polymerase (New England BioLabs, Ipswich, MA, USA); (2) if results were positive for all individuals at a particular reaction condition, PCR subcloning was conducted on each member of the screening set; (3) 10 clones were sequenced on an ABI 3100 Capillary Sequencer (Life Technologies Corporation, Carlsbad, CA, USA); (4) sequences were edited and aligned using Sequencher 4.7 (GeneCodes, Ann Arbor, MI, USA) and exonic regions of each marker were subsequently aligned to the *P. trichocarpa* genome. Of the 18 initial primer pairs, 12 were amplified in each member of the screening panel, but five of these appeared to be members of multicopy gene families (as determined by recovering more than two unique sequences per individual during the PCR subcloning step). One of the remaining loci was discarded because it did not produce clean reads when the PCR product was sequenced, and we selected four of the six remaining loci at random. Nuclear coding genes *LFY*, *NRPB2* and *GLB1*, as well as intron *15int6*, were chosen (Table 1). The selected regions were aligned against the *P. trichocarpa* genome to confirm that the region was indeed single-copy and to ensure that variation would be allelic. As recombination within loci violates the assumptions of the methods used here, which are derived from coalescent theory, the resulting data were screened for recombination using IMgc (Woerner *et al.*, 2007), and sequences that

Table 1 Summary of sequence data collected for *Salix melanopsis* from the Pacific Northwest. Shown for each locus are the total number of individuals sequenced (n), the length of the locus (bp), the number of sites with insertions/deletions (Indel), the per locus estimate of nucleotide diversity (π), the number of segregating sites (SS), Tajima's D (D), and the model of sequence evolution used to estimate the gene trees.

Locus	n	bp	Indel	π	SS	D	Model
15int6	115	674	8	0.994	16	−1.224	F81
GLB1	107	707	37	4.848	16	0.8046	F81 + γ + I
LFY	105	846	19	8.308	25	−1.1435	GTR + γ + I
mat K	127	1159	30	10.111	26	−0.1112	F81 + γ
NRPB2	119	778	30	8.451	35	1.565	HKY + γ + I

showed evidence of recombination were subsequently pruned from the data set for these analyses.

Diversity statistics and gene tree estimation

Diversity statistics were computed in DnaSP 5 (Librado & Rozas, 2009), and models of sequence evolution were selected using DT-ModSel (Minin *et al.*, 2003) (Table 1). After excluding redundant haplotypes using MacClade 4 (Maddison & Maddison, 2011), gene trees were estimated using maximum likelihood as an optimality criterion in paup* 4.0b10 for UNIX (Swofford, 2003).

Species delimitation and population subdivision

Several new methods for species delimitation have recently been introduced. In general, these can be divided into validation methods that are intended to evaluate proposed intraspecific divisions such as subspecies or races (e.g. Rannala & Yang, 2003; Ence & Carstens, 2011) and discovery methods that attempt to identify cryptic diversity in the absence of any a priori ideas regarding species limits (e.g. Pritchard *et al.*, 2000; Pons *et al.*, 2006; O'Meara, 2010). We followed both strategies, and used SpedeSTEM 1.0 (Ence & Carstens, 2011), BP&P 2.0b (Rannala & Yang, 2003; Yang & Rannala, 2010), and structure 2.3.2.1 (Pritchard *et al.*, 2000) to evaluate the proposal by Brunsfeld *et al.* (1992) that *S. melanopsis* be divided into three races. In the BP&P and SpedeSTEM analyses, each race was treated as a putative species, and support for different species trees were evaluated. In the structure analyses, the full data set was used with and without a priori racial divisions, and the resulting likelihoods were compared. While these analyses alone are not sufficient to determine the taxonomic status of *S. melanopsis*, a well-supported finding of cryptic evolutionary lineages within *S. melanopsis* would justify a more thorough exploration of the morphological and environmental divergence of these races.

SpedeSTEM and BP&P are implemented in a species tree coalescent framework (Edwards, 2009) and compare the probability of the sequence data under a model where all samples are members of the same (operational) species with alternative models where the data are divided into two or more species. While these methods differ in their statistical frameworks, they share several underlying assumptions, namely that shared polymorphism is attributable to incomplete sorting of ancestral polymorphism (i.e. no gene flow), and that N_e is constant (i.e. no population expansion or contraction).

SpedeSTEM evaluates alternative models of lineage membership using information theory. For the *S. melanopsis* data, five models of putative lineage delimitation were evaluated: (1) samples were divided into three lineages corresponding to the races (lowland, mesic and subalpine) described by Brunsfeld *et al.* (2007); (2) samples were grouped into a single lineage; and (3)–(5) two of the three races were combined into a single lineage and the third treated as separate (all three combinations were tested). Note that we attempted to generate sequence data from all available samples, and that any difference in the number of mesic, subalpine and lowland samples reflects their representation in the collection. However, because the number of sampled alleles differed among proposed races and loci, we followed Hird *et al.* (2010) and used a replicated subsampling strategy. Using SpedeSTEM, we drew five or ten alleles at random from each putative lineage, computed the species tree using STEM 1.0 (Kubatko *et al.*, 2009) and estimated likelihoods based on each of the five models. Models were compared according to Akaike information criterion (AIC) values. We then repeated this analysis 999 times.

BP&P 2.0b (Rannala & Yang, 2003; Yang & Rannala, 2010) also evaluates a priori models of species relationships, but instead of computing the probabilities of alternative species trees, it returns posterior probabilities for each node in a specific guide tree. Multiple runs are necessary with different guide trees in order to more fully explore species tree space. A data set consisting of only the four nuclear loci was analysed because BP&P does not include a specified inheritance scalar. For the BP&P analysis, we used three different guide trees: (*S. alba*, (lowland, (mesic + subalpine))), (*S. alba*, (mes, (low + sub))) and (*S. alba*, (sub, (low + mes))). The default prior, Gamma (2, 1000), was used for values of θ and τ_0. To confirm the stability of the results, each analysis was run at least five times with Markov chain Monte Carlo (MCMC) acceptance rates ranging from 0.10 to 0.75 for each appropriate fine tuning parameter. Both rjMCMC algorithms 0 and 1 were tried with $\varepsilon = 2$, 5 or 20, $\alpha = 1$ or 2, and $m = 1$. For both the SpedeSTEM and BP&P analyses, sequences from *S. alba* were used as an outgroup.

In addition to the validation methodologies implemented above, we used the program structure 2.3.2.1 (Pritchard *et al.*, 2000) as a discovery tool. To test the proposed racial delimitations, the results of cluster assignments were compared between runs with and without knowledge of racial identity. The program was run with clustering levels (K) fixed at $K = 2$ or $K = 3$. When incorporating race information within the prior distribution, the LOCPRIOR model was used and individuals marked as 'lowland', 'mesic',

'subalpine' or 'unknown'. Also, all subgroupings of races were tried for $K = 2$ (e.g. individuals were marked as 'lowland', 'not lowland' or 'unknown'; this was repeated for the subalpine and mesic races). All runs were repeated 10 times. Log-likelihood scores were compared using an AIC framework among runs with and without the LOCPRIOR model to assess the validity of dividing the samples by race.

Evaluating models using approximate Bayesian computation

We chose to use approximate Bayesian computation (ABC) rather than a hypothesis-testing framework to evaluate the support for specific demographical models. Given the number of data collected here, estimates of θ and divergence times necessary for both frequentist (e.g. Knowles, 2001) and Bayesian (e.g. Fagundes *et al.*, 2007; Peter *et al.*, 2010) hypothesis-testing approaches are likely to be inaccurate (Felsenstein, 2006; Carling & Brumfield, 2007), particularly because the underlying demographic history of the focal taxa is unknown. ABC attempts to integrate across this uncertainty in parameter values (Csilléry *et al.*, 2010), and thus is probably more appropriate for evaluating the relative support of multiple models given the data than a hypothesis-testing framework. ABC approximates the posterior distribution of a model based on summary statistics without having to compute the full likelihood (Beaumont *et al.*, 2002).

Approximate Bayesian computation (Pritchard *et al.*, 1999; Beaumont *et al.*, 2002) was used to explore the fit of demographic models to the data in two ways. First, we evaluated the fit of seven demographic models suggested by previous work in this system (Fig. 1). Models included 'ancient vicariance' and 'recent dispersal' (Brunsfeld *et al.*, 2001), as well as a model describing two glacial refugia in the northern Rocky Mountains – in the Clearwater and Salmon River regions (Brunsfeld *et al.*, 2007). Second, we explored the fit of our data to several commonly assumed coalescent-based models of population expansion, migration and isolation with migration. Data were simulated under models that matched the default settings implemented in LAMARC (Kuhner, 2006), and simplified models in MIGRATE-N (Beerli & Felsenstein, 1999, 2001) and IMA (Hey & Nielsen, 2007), and the fit of these models to the true data was assessed. Simplifications for MIGRATE-N and IMA were symmetrical migration rates and constant θ.

For both the ABC analyses involving the seven demographic models and the three coalescent models, we used the approach first described by Pritchard *et al.* (1999), as follows.

1. A prior distribution was constructed by simulating sequence data sets under each of the possible models. For instance, for the demographic model test, MS (Hudson, 2002; version released 2007) was used to simulate sequence data under each of the seven models described in Fig. 1. The simulated data matched the empirical data set in terms of the number of loci and the representation of samples in different regions. A custom PERL script was used to generate 1,000,000 simulated data sets for each model.

2. The posterior distribution was estimated by retaining the 0.0005% of data sets most similar to the empirical data. Similarity was determined by calculating the Euclidean distance between summary statistics of the simulated and empirical data sets. The choice of the particular summary statistic vector used is described more fully below, but possible vectors included nucleotide diversity (π_{Tot}), the number of segregating sites (SS), Tajima's D (D), nucleotide diversity within populations (π_w), and nucleotide diversity between populations (π_B). The software SAMPLE STATS (Hudson, 2002; version released 2007) was used to calculate the summary statistics, and MSREJECT 20100519 (Hickerson *et al.*, 2007) was used to conduct the rejection step and construct the posterior.

3. The posterior probability of a given model is measured by its proportional representation in the posterior distribution.

For the ABC analysis of the seven demographic models, parameters were drawn from the following prior distributions: $\theta = 4N_e\mu$ (where N_e is the effective population size and μ is the mutation rate) was drawn from a uniform prior over the range 0.01–10.0 for all models; divergence times were drawn from uniform distributions of T_b = 2.001–8.0 (for the pre-Pleistocene), T_m = 0.5001–1.0 (for the mid- to late Pleistocene) and T_p = 0.0001–0.5 (for the post-Pleistocene); and the intrinsic growth rate, γ, was drawn from an exponential distribution over 0.001–3.0.

The specific summary statistic vector used in the rejection step can have an impact on evaluating which model is most probable. To assess which summary vector was most likely to identify the model with the best fit to the data we performed additional simulation testing: 100 data sets were randomly chosen from the simulated data sets for each of the seven models and were treated as if they were the empirical data. For each of these 'known' data sets, we used MSREJECT to calculate the posterior probability of each model using the prior distribution generated above. We tried 24 different summary vectors in generating the posteriors, and selected the vector that maximized the probability of the true model while minimizing the average probabilities of the false models.

The ABC analysis was then repeated using models that matched those of the common coalescent-based programs LAMARC, MIGRATE-N and IMA. Priors on parameter values were uninformative and as follows: θ = 0.01–12.0; τ = 0.01–3.0; M = 0.01–3.0; γ = 0.01–1.0. In order to minimize bias in the ABC analysis favouring models with fewer parameters, only a single migration parameter (i.e. symmetric migration rates) and a single θ parameter were used in the MIGRATE-N and IMA analyses.

For the sake of comparison, we then analysed our empirical data using these software packages as follows. LAMARC was used to estimate the intrinsic population growth rate (γ) and θ using maximum likelihood; the analysis was conducted using 20 initial chains and two final chains, with the model of sequence evolution for *LFY* set to GTR and the rest set to F84; the inheritance scalar for *mat*K was set to 0.25 (other

regions were set to 1), and other program parameters set to default values. MIGRATE-N analyses were conducted using maximum likelihood with migration allowed between the Cascades, Clearwater and Salmon River regions; in general, run conditions matched those described in Koopman & Carstens (2010) and models of sequence evolution were as close as possible to those described in Table 1. The default fully parameterized model in IMA was also used to estimate divergence and gene flow between the Cascades and northern Rocky Mountain populations. We coupled 20 Markov chains in a geometric heating scheme (–ha 0.05, –hb1.0), included a burn-in of 100,000 steps, and ran the chains until the effective samples sizes of all parameters exceeded 50 and there were no visible correlations in the trend plots. An HKY model was assumed, and after exploration, runs were conducted using the following priors ($\theta = 25$; $M = 5$; $T = 20$). Because Brunsfeld *et al.* (2007) proposed that there were two northern Rocky Mountain refugia during the Pleistocene, we also divided the samples from the northern Rocky Mountains into the Clearwater and Salmon River regions and repeated the analysis using settings that matched the above.

Table 2 Results from a SPEDESTEM analysis of *Salix melanopsis* where racial delimitations in the Pacific Northwest were tested. Analyses were carried out by subsampling five or ten alleles/race. *Salix alba* was used as an outgroup.

Model	No. of bests	wAIC
(Five alleles/race)		
Alba(Low, Mes, Sub)	54	0.1158
Alba(Low + Mes + Sub)	256	0.1799
Alba(Low(Mes + Sub))	427	0.4112
Alba(Mes(Low + Sub))	102	0.1420
Alba(Sub(Low + Mes))	161	0.1510
(Ten alleles/race)		
Alba(Low, Mes, Sub)	116	0.1245
Alba(Low + Mes + Sub)	293	0.1824
Alba(Low(Mes + Sub))	315	0.3385
Alba(Mes(Low + Sub))	132	0.1725
Alba(Sub(Low + Mes))	144	0.1821

Races are: Low = lowland; Mes = mesic; Sub = subalpine.
No. of bests, number of times out of 1000 replicates that the model was chosen as the best model; wAIC, weighted Akaike information criterion values for that model averaged across replicates.

RESULTS

The *Salix melanopsis* data set

In total, 145 samples of *S. melanopsis* were collected from 33 localities, 19 river drainages and from across the range in the Cascades and northern Rocky Mountains, including putative refugia in the Clearwater and Salmon River basins (Table S1). Individuals were sampled from each of the three hypothesized races: lowland, mesic and subalpine. Four nuclear loci were sequenced and additional samples were added to the previously developed chloroplast *mat*K data set. Genetic diversity was high (Table 1). Sequences were deposited in GenBank with accession numbers: JX187155–JX187361.

Species delimitation

The SPEDESTEM and BP&P analyses found little support for the proposal by Brunsfeld *et al.* (2007) that *S. melanopsis*, as currently described, contained three races. Using SPEDESTEM, the model that treated each race as a separate evolutionary lineage was ranked highest in little more than 5% of the replicates when subsampling five alleles/race and in approximately 12% of the replicates in the 10 alleles/race analysis, and the three-race model had the lowest overall model probability (Table 2). However, there was some indication that the lowland race was an independent lineage, as the weighted AIC of this model was ranked as best in the SPEDESTEM replicates. Multiple runs of BP&P with different MCMC parameters and guide trees converged on similar results (Fig. 2), where *S. alba* was found to be distinct from *S. melanopsis* with high support (posterior probability, PP = 1.0), but nodes separating any single race from another were completely collapsed (PP < 0.0005). Trees that had each race as an independent lineage had no support in any run (PP = 0.00000).

STRUCTURE runs incorporating the racial identity of individuals in the prior performed worse than runs with uniform priors (Fig. 3), indicating that racial divisions were incongruent with the population clusters. The subalpine and lowland individuals were most often assigned to the blue genetic cluster (Fig. S1), but the mesic race contained many representatives of this blue cluster as well. The genetic clusters were all widespread among localities, but the orange cluster ($K = 2$ and $K = 3$) was at higher frequencies in the Clearwater drainage of the northern Rocky Mountains (Appendix S2). At $K = 3$, the blue genetic cluster was divided into two widespread groups, one with higher frequencies in the Cascades (Appendix S2).

Using ABC to evaluate biogeographical models

Simulation testing indicated that the vector that incorporated all summary statistics (SS, D, π_{Tot}, $\pi_{w,nrm}$, $\pi_{w,cas}$, π_B) was the most effective at identifying the true historical model (Table S2). Using this vector, the single population expansion model (model 6) had the highest posterior probability ($Pr_{Model\ 6} = 0.684$) followed by the compartmentalized refugia with a southern source for Cascades populations ($Pr_{Model\ 2} = 0.246$; Table 3). No other model had a posterior probability higher than 0.05 (Table 3). These results support recent expansion from a single refugium.

The ABC analysis of LAMARC, MIGRATE-N and IMA models

Simulation testing indicated that the summary statistic π_{Tot} was most effective at identifying the true demographic model

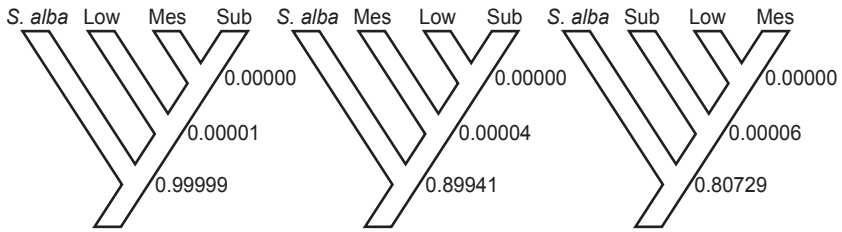

Figure 2 Results from the BP&P analysis where racial delimitations were tested for *Salix melanopsis* sampled in the Pacific Northwest. For each guide tree, posterior support for each node in the species tree is shown. Posterior probabilities were averaged across all runs. Low = lowland, Mes = mesic, and Sub = subalpine races. *Salix alba* was used as an outgroup.

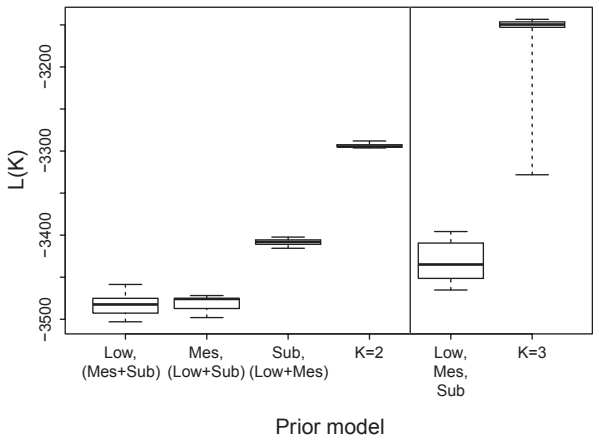

Figure 3 Log-likelihoods of population clustering analyses, L(*K*), in *Salix melanopsis* from the Pacific Northwest based on different race-based priors. Individuals were assigned to two (left) or three (right) population clusters using the Bayesian assignment program STRUCTURE. Priors were based on the individuals' racial identity (Low = lowland, Mes = mesic, Sub = subalpine) or were uniformly distributed across two or three populations ($K = 2$ or $K = 3$, respectively). The bold horizontal line shows the median log-likelihood value from 10 runs; boxes outline the 25% and 75% percentiles; whiskers extend to the minimum and maximum values.

(Table S3). Using this vector, we found that the single population with expansion model implemented in LAMARC was a better fit to the data than the models including migration implemented in MIGRATE-N and isolation with migration in IMA (Pr_{LAMARC} model = 0.93; $Pr_{MIGRATE\text{-}N}$ model = 0.00; Pr_{IMA} model = 0.07). We then used LAMARC to estimate $\theta = 45.1776$ (95% CI 41.6–49.088) and the exponential growth rate $\gamma = 351.2$ (271.5–430.7). Values of θ estimated using MIGRATE-N were generally smaller and are indistinguishable between the Cascades, Clearwater and Salmon River regions (θ_{Cas}, θ_{Cle}, $\theta_{Sal} \approx 7.98$); smaller θ values from MIGRATE-N are expected in cases of population growth because they are time averaged versus the present-day θ estimated by LAMARC. Effective migration rates between regions estimated using MIGRATE-N have largely overlapping confidence intervals but with elevated migration from the Clearwater to Salmon River regions ($m_{Cle \rightarrow Sal} = 4.70$, $m_{others} = 1.06$–1.99; Table S4). Estimates of effective migration rates between regions from the corresponding IMA analysis were of the same order of magnitude ($m = 1.01$–8.46) with higher migration rates from the Clearwater to Salmon River regions ($m_{Cle \rightarrow Sal} = 8.46$) and from the Cascades to the northern Rocky Mountains ($m_{Cas \rightarrow Cle+Sal} = 5.56$; Table S5).

Table 3 The fit of various historical demographic models to the five loci data set of *Salix melanopsis* as assessed by an approximate Bayesian computation analysis. The posterior probabilities (PP) of each model (illustrated in Fig. 1) are shown. The models are: (0) ancient vicariance between the Cascade Range and northern Rocky Mountains, (1) ancient vicariance followed by differentiation into compartmentalized Rocky Mountain refugia in the Clearwater and Salmon River regions, (2) compartmentalized refugia in the Rockies followed by colonization of the Cascades from the southern Salmon River region, (3) compartmentalized refugia followed by colonization of the Cascades from the northern Clearwater region, (4) a single refugium, post-Pleistocene dispersal to the Cascades, (5) a single refugium, mid-Pleistocene dispersal to the Cascades, (6) a single population with expansion beginning in the mid-Pleistocene and continuing to the present.

Model	Parameters	PP
(0) Ancient vicariance	θ, T_b	0.0
(1) Anc. vicariance, comp. refuge	θ, T_b, T_m	0.0
(2) Comp. refuge, S source	θ, T_m, T_p	0.246
(3) Comp. refuge, N source	θ, T_m, T_p	0.0
(4) One refuge, T_p dispersal	θ, T_p	0.053
(5) One refuge, T_m dispersal	θ, T_m	0.018
(6) One popn, T_{m-p} expansion	θ, $T_{m\text{-}p}$, γ	0.684

Parameters: θ, $4N_e\mu$; T_b, divergence time before the Pleistocene; T_m, divergence time mid-Pleistocene; T_p, divergence time post-Pleistocene; T_{m-p}, timing of expansion mid-Pleistocene to present; γ, intrinsic growth rate.

DISCUSSION

One of the most basic assumptions required of any phylogeographical investigation is related to the set of samples to include in the analysis. In the case of *S. melanopsis* it was unclear whether we should analyse all samples identified as members of this species or only those described as belonging to the mesic race. To a large extent, our work was motivated by the proposal by Brunsfeld *et al.* (1992) that *S. melanopsis* was composed of three races that were adapted to lowland,

mesic and subalpine habitats, respectively. This hypothesis was supported by a chloroplast (*mat*K and *rpl*16) gene tree (Brunsfeld *et al.*, 2007); however, the analysis only included 12 specimens and had low maximum likelihood bootstrap support for the mesic race (Brunsfeld *et al.*, 2007). We re-tested this proposal using an expanded dataset and species tree methods such as SpedeSTEM and BP&P, which have recently been used to recognize cryptic lineages in *Myotis* bats (Carstens & Dewey, 2010) and African geckos (Leaché & Fujita, 2010). The results indicate that the races proposed by Brunsfeld *et al.* (1992) are not distinct evolutionary lineages. None of the species delimitation analyses supported a model where the three races represented independent lineages (Table 2, Fig. 2). This suggests that *S. melanopsis* exhibits morphological plasticity that manifests itself in the lowland, mesic and subalpine habitats. Alternatively, our data may be insufficient to detect these lineages. However, the number of loci collected here was similar to that used for delimitation in the geckos and bats (Carstens & Dewey, 2010; Leaché & Fujita, 2010), and results from a structure analysis indicate that the likelihoods of the proposed race delimitations are far lower than the likelihoods of optimal *K* levels (Fig. 3). Taken together, we do not feel that the collection of additional sequence loci is justified for this particular question. We interpret our results as an indication that *S. melanopsis* in the mesic forests represents a single evolutionary lineage, and analysed the data without regard to their racial assignment in the other analyses.

Despite this finding, our data contain considerable signal regarding the demographic history of *S. melanopsis*. In general, this signal is consistent with the end-Pleistocene environmental niche model for *S. melanopsis* developed by Carstens & Richards (2007), which described a contiguous region of suitable habitat that encompassed both the Clearwater and Salmon River drainages during the Last Glacial Maximum (LGM), and predicted a lack of suitable habitat in the Cascades. The dominant signal in our data suggests that *S. melanopsis* expanded from a single refugial population at the beginning of the Holocene:

1. In our ABC analysis that evaluated existing phylogeographical hypotheses, most of the posterior probability (*c.* 68%) was represented by a single-refugium expansion model (i.e. model 6 in Fig. 1; Table 2).

2. When we evaluated the relative fit of our data to the models implemented in IMa, Migrate-n and lamarc, most of the posterior probability (93%) was represented by the single-population expansion model used in lamarc (Table 4). When lamarc is used to analyse our data, estimates of the exponential rate of increase, γ, are high and the confidence interval excludes zero.

3. Other analyses that are not based on explicit coalescent models also appear to be consistent with this inference. For example, we tested for isolation by distance by comparing genetic distances and geographical distances among all 33 localities, and found that these distances were correlated ($r = 0.442$, $P = 0.0003$).

Table 4 The fit of commonly used coalescent-based models to the five loci data set of *Salix melanopsis* from the Pacific Northwest as assessed by an approximate Bayesian computation analysis. The posterior probabilities (PP) and parameters of each model are shown.

Model	Parameters	PP
Lamarc	θ, γ	0.93
Migrate-n	θ, m	0.00
IMa	θ, m, t	0.07

θ, $4N_e\mu$; m, migration rate; t, divergence time; γ, intrinsic growth rate.

One aspect of our data that is seemingly at odds with this model is the relatively high amount of genetic diversity present within *S. melanopsis* (Table 1). Upon initial examination, we suspected that this was too high for a species that was compressed into a single Pleistocene refugium. This was in part based on comparisons with vertebrate species such as *Plethodon idahoensis* and *Dicamptodon aterrimus*; previous work suggested that these species expanded from small glacial refugia in the northern Rocky Mountains (Carstens *et al.*, 2004, 2005a). However, the niche models of Carstens & Richards (2007) imply that the ranges of both *P. idahoensis* and *D. aterrimus* were greatly reduced at the LGM, while the range of *S. melanopsis* was far more extensive. Also, the expansion from a single refugium model for *S. melanopsis* was supported by a posterior probability of only 0.68, which suggests that better models may exist. The level of diversity present in *S. melanopsis* could indicate a complexity to the refugial dynamics that is not captured by the simple model implemented in the ABC and lamarc analyses. In total, these results support the suggestion of Shafer *et al.* (2010) that refugial dynamics in the Pacific Northwest were more complex than is commonly appreciated.

Phylogeographical inferences should not be based solely on parameter estimation, because inferences derived from parameter estimates can be misleading when based on poorly fitting models. For example, a Cascades–northern Rocky Mountains partitioning of samples has been used by previous phylogeographical investigations into *S. melanopsis* and other co-distributed taxa (e.g. Brunsfeld *et al.*, 2001; Carstens *et al.*, 2005b), so it may appear reasonable to adopt an isolation-with-migration model using this east–west division without first testing model fit. If this is done, and we adopt the mutation rate estimated for *Arabidopsis thaliana* by sequencing *de novo* mutation lines (7.0×10^{-9}; Ossowski *et al.*, 2010), the timing of divergence between the Cascades and the northern Rockies is estimated to be approximately 200,000 generations, or well into the Pleistocene, with relatively low rates of migration ($< 2.5 \times 10^{-6}$). These parameter estimates would support an ancient vicariance scenario (model 0) even though this model was clearly not favoured by our ABC analysis of historical models (Tables 2 & 3). The magnitude of these values would have been easy to misinterpret had we not assessed the fit of the

isolation-with-migration model to the data. These results indicate that researchers who are interested in analysing their data with pre-existing software packages should consider the fit of the models implemented in these packages to their data. One approach to do so is represented by the model evaluation ABC analysis described here, but as a discipline we currently lack the phylogeographical equivalent of MODELTEST (Posada & Crandall, 1998) for quickly determining which of several implemented models is an optimal fit to the data.

Recent authors (Camargo *et al.*, 2010; Garrick *et al.*, 2010) have emphasized the importance of congruent signal across methods; for *S. melanopsis*, expansion from a single Pleistocene refugium is supported by many analyses (ABC of historical models, LAMARC, isolation-by-distance, etc.), and we interpret this congruence as evidence of the accuracy of this inference. Consequently, we follow other recent authors (Beaumont *et al.*, 2010; Csilléry *et al.*, 2010; Hickerson *et al.*, 2010) in suggesting that ABC is a valuable addition to the phylogeography toolbox. While there are certainly challenges associated with this approach to data analysis (e.g. demographic model space may be difficult to parameterize, and it may be difficult to find a good summary statistic vector that represents the empirical data), ABC is one of few methods that can be used to quantify the relative fit of explicit demographic models to the data. However, we also suggest that ABC, at least as implemented here, has some shortcomings. One is that the use of insufficient summary statistics can lead to a loss of information that can bias the results (Robert *et al.*, 2011). We performed simulation testing, which indicated that using a vector composed of all statistics was optimal for the historical models, but that π was adequate to differentiate the models implemented in IMA, LAMARC and MIGRATE-N. A related issue concerns differences in the dimensionality of the compared models: differences in the number of parameters can bias the ABC analysis in favour of models with fewer parameters. By simplifying our LAMARC, MIGRATE-N and IMA models to two, two and three parameters, respectively, and by comparing historical models with similar numbers of parameters, we hoped to minimize this bias.

CONCLUSIONS

Our research highlights the importance of using models to explore evolutionary processes. Based on an analysis of five loci from 145 *S. melanopsis* individuals, we find little support for the intra-specific racial divisions proposed by Brunsfeld *et al.* (1992). However, our data contain a strong signal regarding the demographic history of *S. melanopsis*. They indicate that the species probably colonized the western portion of its range following expansion from a single Pleistocene refugium. Additionally, the high levels of genetic diversity may indicate that *S. melanopsis* did not pass through a severe bottleneck, as other co-distributed species did.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supplementary sampling information and results (Tables S1–S5 and Fig. S1).

Appendix S2 *Salix melanopsis* GIS data (KMZ file openable in Google Earth).

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BIOSKETCH

This study is part of ongoing research in the lab of **Bryan Carstens** that seeks to investigate the post-glacial dynamics of the Pacific Northwest and develop novel methods for species delimitation. He and post-doctoral fellow **Yi-Hsin Erica Tsai** share an interest in improving comparative phylogeographical methodologies. Her other work has centred on the community assembly of parasitic plants and eastern North American trees, while the Carstens Lab also investigates the community phylogeography of pitcher plants and species limits in *Myotis* bats, as well as the development of bioinformatics tools.

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