TWO CRYPTIC SPECIES WITHIN *ASTRAGALUS CUSICKII* DELIMITED USING MOLECULAR PHYLOGENETIC TECHNIQUES

by

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ABSTRACT

Understanding the source of phenotypic variability is a challenge in the biological sciences. Variation in phenotypes is the result of variation in the genetics and environment the organism experiences, but elucidating the relative contribution of these two parameters can pose problems, especially in the field of systematics. Systematists are challenged to classify biological diversity into groups that share common ancestry. Phenotypic variation can be useful to demonstrate common ancestry, but only when the primary contributor to the variation is under strong genetic control, and thus heritable. Cusick's milkvetch (*Astragalus cusickii*) is a perennial forb endemic to the intermountain west region of the United States. The species currently comprises four varieties based on subtle morphological dissimilarities, such as leaf size and density, and the size and shape of the seed pods. The taxonomic organization of the varieties of *A. cusickii* and related species of *Astragalus* were reexamined through phylogenetic analysis of nuclear, nuclear-ribosomal, and chloroplast gene regions. Maximum parsimony, maximum likelihood, Bayesian inference, the genealogical sorting index, an approximately unbiased test, and multispecies coalescent analysis were used to determine appropriate species boundaries under the phylogenetic species concept. The results support reclassification of *A. cusickii* var. *packardiae* and *A. cusickii* var. *sterilis* as separate species. Additionally, evidence suggests a chloroplast capture event may have occurred in one population of *A. cusickii* var. *packardiae*.

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INTRODUCTION

Our understanding of the biological world, among scientists and non-scientists alike, is founded in the grouping of organisms into categories based on observations of phenotypic variation. When we distinguish one group of organisms from another, be they from different kingdoms or from the same species, we begin with the observation of differences in phenotypic characteristics. The immediately apparent phenotypic differences between a mammal and a magnolia tree are on a spectrum with the more subtle phenotypic traits used to conceptually separate one breed of dog from another. The consideration of phenotypic variation is crucial to any understanding of the diversity of life. However, phenotypic variation alone is not an unambiguous criterion for designating species. While we may be comfortable applying labels to breeds of dog based on criteria such as differences in coat color, few would argue that different breeds should be considered separate species.

Species Concepts

The concept of species is fundamental to the study of biology, and represents one of the most important operational units used by biologists (de Queiroz, 2005; Mayr, 1982). Species provide one of the central pillars of the conceptual framework within which much biological research is conducted. Perhaps more importantly, the concept of species is one of the few biological terms widely, if not always accurately, understood by those outside of the discipline of biology, providing biologists with a crucial tool for communicating with the public about the implications of biological research.

Observable variation in phenotypes is an important aspect of biology, yet it is inadequate to fully explain one of the central concepts of the discipline: species. For all its undeniable importance, finding a discipline-wide consensus as to the precise definition of the concept of species has proved difficult, and has provoked considerable discussion. In their attempts to delimit particular species boundaries, biologists have created many competing definitions of species, including at least 24 formally named species concepts, many of which are mutually exclusive, and provide for differing boundaries between species, and different counts of species totals within various genera (de Queiroz, 1998, 2007; Harrison, 1998; Mayden, 1997).

Probably the most commonly understood definition of species comes from the biological species concept. Under this concept, species are defined by the ability or potential of organisms to reproduce, resulting in fertile offspring (Dobzhansky, 1950; Mayr, 1942; Wright, 1940). This approach has several advantages. Among these, it is perhaps the most immediately intuitive concept of species, particularly to non-biologists. Whereas the other species concepts may require a background in biology to fully appreciate, understanding that only organisms of the same species can interbreed, could almost be thought of as conventional wisdom. It is a simply stated criterion, and provides for a fairly straightforward test (in some cases). The biological species concept also has the advantage of aligning with our conceptual understanding of populations as interbreeding groups of individual organisms.

However intuitive the biological species concept may appear to be at first glance, a fairly long list of exceptions to the rule exists. Many organisms do not reproduce sexually, for example, and so become difficult to define. In a species in which all

individuals are born as pregnant females, each essentially giving birth to her own clones, should we consider every individual lineage as a separate species, no matter how much similarity in morphology or ecological niche they share? Ring species, in which adjacent populations can interbreed, but more distant populations cannot, pose another challenge for the biological species concept. Such species present a paradox: By the biological species concept, the adjacent populations which can interbreed form a single species, yet under the same concept the more distantly placed populations which cannot interbreed are not members of the same species. There are also significant concerns as to the practicality of the concept when applied to field research. In many cases it may be difficult or impossible to verify the interbreeding, or even potential of interbreeding, among groups of organisms (Skokal and Crovello, 1970). In such cases, the biological species concept, even if useful in the abstract sense, becomes meaningless in practice.

Hybrids present another example of a situation where the biological species concept is not a good model to explain the boundaries between species. In an example which illustrates this phenomenon, Clay et al. (2012) present a situation in *Castilleja*, where two species with overlapping territory form a hybrid species. The hybrid species exhibits a morphology unique from both of the progenitor species. All three species, progenitors and hybrid, were demonstrated to be following independent evolutionary trajectories. The three taxa would each separately meet the criteria of species under many of the alternative species concepts, yet due to each being reproductively compatible with the others, the biological species concept would consider all three taxa as a single species.

Even when applied to organisms that reproduce sexually, and are not ring species or hybrids, the biological species concept can still lead to results that are not reflective of

the true genealogical history of the organisms in question. Often, particularly in cases in which a single population becomes reproductively isolated from other populations which remain reproductively compatible with each other, the biological species concept can lead to a group of organisms being labeled as a single species, but which actually represent a paraphyletic evolutionary history (Velasco, 2008). This in turn can lead to scenarios where species trees generated under a framework of the biological species concept fail to show true, historical genealogical relationships among taxa, or worse, show incorrect relationships. The biological species concept defines the boundary between species as reproductive isolation, but reproductive isolation is only one of many character changes that will occur between taxa during the process of speciation. It is not necessary that reproductive isolation be the first of these character changes to occur (de Queiroz, 1998). It is then possible to imagine a scenario where two recently diverged populations have evolved to become reproductively isolated from one another, yet one retains reproductive compatibility with a more distantly related population as a symplesiomorphy (Velasco, 2008). In this case, the biological species concept would define the more distantly related populations as a single species due to their retained reproductive compatibility, even though one of the populations actually shares a more recent common ancestor with the now reproductively isolated population. The error of species defined by the biological species concept representing paraphyletic groups occurs frequently enough to preclude dismissing the problem as too rare to be important. Funk and Omland (2003) surveyed 584 animal phylogeny studies, and found evidence of paraphyly in 23% of the species included in the studies in which detecting paraphyly was possible. Their findings demonstrate that the errors resulting from defining species based on reproductive

isolation have a real and negative effect on our understanding of the actual historical relationships among groups of organisms.

An alternative to the biological species concept is the phylogenetic species concept, in which species are defined as the smallest group of organisms that share a common ancestor, and which are distinguishable from other such groups (Donoghue, 1985). This approach avoids many of the potential problems with the biological species concept by focusing directly on the historical genealogical relationships among taxa. Asexually-reproducing organisms do not present a problem for the phylogenetic species concept. Individuals from asexually-reproducing taxa can be defined as belonging to a species, assuming shared morphology (though determining the degree of morphological differentiation which will define separate species may become a problem – see below). Likewise, ring species do not present the phylogenetic species concept with the same paradox encountered under the biological species concept. A ring species, containing populations at the extremes (often geographic extremes) which are reproductively isolated from one another, would be defined as a single species as long as all populations possess the same most recent common ancestor, and are morphologically diagnosable from other groups of organisms. The phylogenetic species concept also avoids the problems of paraphyly encountered as an apparently common outcome of studies employing the biological species concept by focusing directly on the historical genealogical relationships among taxa. In this context, the importance of reproductive isolation is secondary to the historical genealogy. Two populations which are reciprocally monophyletic and morphologically distinguishable from one another would be defined as separate species under the phylogenetic species concept, even if they retained

reproductive compatibility. However, the phylogenetic species concept is not without its flaws. A common criticism is that a rigid interpretation of the concept leads to over-splitting of taxa into too many species. Under certain circumstances, new ‘species’ may arise from a species approaching extinction, as genetic drift in small, isolated populations may give rise to diagnosable morphological differences in populations which share a recent common ancestor (Zachos and Lovari, 2013). At a certain point, diagnosable morphological differences may become arbitrary, particularly when considering the addition of vast amounts of genetic data that can increasingly be considered when comparing taxa.

Objections to the more extreme predictions of the phylogenetic species concept, like similar objections to certain predictions of the biological species concept, illustrate the fact that many biologists have an intuitive understanding of what it is they mean when they speak of ‘species,’ even if the concept is not explicitly defined in their own minds. De Queiroz (2007) has attempted to define this commonly-held, yet elusive, understanding among biologists. According to de Queiroz (2007), all contemporary species concepts have a common vision of what constitutes a species: a separately evolving meta-population lineage. The various competing definitions of species differ only in what secondary criterion is added to this understanding, even if the phrase ‘separately evolving meta-population lineages’ is not actually stated. For example, the biological species concept implicitly defines a species as a separately evolving meta-population lineage which is reproductively isolated from other such groups. Similarly, the phylogenetic species concept defines, though not explicitly, a species to be a separately evolving meta-population lineage with a most recent common ancestor, which is

morphologically diagnosable from other such groups. In what he terms the unified species concept, de Queiroz (2007) asserts that the only appropriate criterion to determine species is whether or not a given group of organisms are a separately evolving meta-population lineage. All other criteria from previous species concepts can be thought of as milestones along the process of speciation, which need not occur in any particular order (de Queiroz, 1998). Under this concept, two diverging groups of organisms could be defined as separate species earlier in the speciation process than under some of the other species concepts – as early as they could be determined to be separately evolving lineages.

An obvious criticism of the unified species concept is that it is too vague. By what criteria, exactly, are groups of organisms determined to be separately evolving meta-population lineages? For good reason, there is a strong bias within the scientific disciplines to define *a priori* as many components of an experiment as possible before beginning. There are several logical reasons for this approach to be an established tradition among scientists, probably most importantly as an attempt to ameliorate the effects of bias on the outcome of an experiment. This impulse would understandably lead many to expect an explicit suite of criteria for defining species to be rigidly followed in all cases across all kingdoms of life. As frustrating as it may be, the vast diversity of life forms on earth may require a definition of species as vague as that of the unified species concept, if it is to be applied across the entire Tree of Life. It may be necessary to accept that if a common understanding of what constitutes a species is to be found among mammals and protozoa, insects and angiosperms, that the definition will have to be broad. It seems likely to be incumbent upon researchers from all sub-disciplines within

biology to determine which criteria for identifying independently evolving meta-population lineages best apply to the organisms they study.

Phenotypic Plasticity

Much of the taxonomic work conducted since the advent of systematics has been performed by carefully examining and comparing phenotypic variation (Hillis, 1987). Traits which are found to be synapomorphies can provide insight into the relationships among taxa. The phenomenon of phenotypic plasticity, in which environmental factors affect the phenotype of an organism, erodes the reliability of phenotypic variation as a method of diagnosing species boundaries. Phenotypic plasticity introduces considerable uncertainty as to which morphological characters are reliable synapomorphies. Phenotype is determined by a combination of genotype and environmental factors. In humans, height is such a phenotype. The height a person reaches at maturity is determined not solely by the individual's genes, but is also heavily influenced by environmental factors such as nutrition (Visscher, 2008).

The morphological effects of phenotypic plasticity are even more pronounced among plants. Being sessile, plants must contend with the environmental fluctuations of the location in which they germinate. Consequently, plants exhibit large-scale physiological responses to variations in environmental factors such as soil nutrient content, temperature, and water availability which can have profound effects on their phenotypic variation (Gurevitch et al., 2002). An example of this phenomenon is heterophylly demonstrated by many aquatic plants in response to fluctuating water availability (Lin, 2002). Such species exhibit conspicuous differences in leaf morphology under different conditions. Separate populations of a particular species of plant living

under different environmental conditions can appear markedly different. The significance of observations of phenotypic variation among individuals or populations, especially among closely related taxa, is therefore brought into doubt. Subtle phenotypic variation between taxa existing in different environmental conditions is a poor criterion for evaluating species boundaries.

Molecular Systematics

Molecular systematics has provided increasingly powerful tools for evaluating species boundaries, and in the study of the evolutionary history of life more broadly. Arguably, the most useful contribution of molecular data to systematics is the vast increase in the number of potentially informative characters. Determining monophyly through morphological analysis requires a difficult search for synapomorphies. There is a relatively small set of describable morphological characters in any organism, no matter how carefully examined or how observant the researcher. The occurrence of homoplasy further compounds this problem. Molecular techniques allow sequences of homologous genes to be used as character states to be compared among taxa in the search for synapomorphies. The number of potentially comparable character states is then greatly increased, as compared to a traditional morphological analysis. From the earliest days of molecular systematics, the power of including DNA sequences to increase the dataset available for analysis was recognized (Hillis, 1987).

Molecular tools are particularly useful when attempting to define the species boundaries between closely related, morphologically similar taxa. *Pseudopontia paradoxa* (ghost butterfly) was divided into two subspecies, indistinguishable from one another but for a subtle morphological dissimilarity: the form of a single hind wing vein.

Molecular phylogenetic analysis demonstrated this understanding to be an underestimation of the diversity of *P. paradoxa*, which was found to contain at least five reciprocally monophyletic groups (Mitter et al., 2011). Under closer observation, additional distinguishing morphological characteristics were found for some of the newly discovered monophyletic groups, such as unique patterns visible on the wings under UV light. While it is possible that these obscure distinguishing features would have eventually been found, it is clear that the discovery of monophyletic groups within *P. paradoxa* by molecular means led to additional scrutiny that made their discovery more likely. In this way molecular data can augment morphological data.

Just as molecular phylogenetic analysis can demonstrate an underestimate of species diversity, the same techniques can demonstrate that our estimates of species diversity within a given genera may be too broad. Taxa previously understood to be separate species may not merit recognition as such. Molecular phylogenetic analysis of *Anticlea vaginata* and *A. elegans*, previously understood to be separate species on the basis of morphology, found that the two taxa are more appropriately classified as members of the same species. *Anticlea vaginata* is now classified as *A. elegans* subsp. *vaginata* (Palmquist et al., 2015).

Combined, molecular phylogenetic techniques and the phylogenetic species concept provide researchers with the ability to delimit species boundaries with a level of precision previously unavailable. The use of nucleotide sequences as characters for comparison among taxa allows for criteria more closely aligned with the framework of the phylogenetic species concept by determining directly which taxa represent monophyletic lineages. This approach considers phenotypic variation in a larger context.

When occurring simultaneously with reciprocal monophyly as demonstrated by molecular methods, phenotypic variation can be understood as likely representing inherited, genetically-based traits. Phenotypic variation without demonstration of reciprocal monophyly is often not suitable to define species.

Study Species

The genus *Astragalus* is a highly diverse group of legumes, containing more recognized species than any other genus among the flowering plants (Frodin, 2004; Lock and Schrire, 2005; Mabberley, 2008). Until recently many systematicists regarded *Astragalus* as a 'wastebasket' genus, likely to be paraphyletic (Polhill, 1981; Wojciechowski, 2005). Many previously described groups of organisms have undergone considerable rearrangement as the application of modern molecular techniques within the field of systematics has demonstrated them to actually be paraphyletic or polyphyletic groups. However, the monophyly of *Astragalus* has been well-supported (Sanderson, 1991; Sanderson and Doyle, 1993; Wojciechowski et al., 1993, 1999). Many species-level relationships within the genus remain unresolved. The large geographic distribution and extreme diversity of *Astragalus* make it a difficult genus for systematic studies (Scherson et al., 2005). Mating systems have been studied in fewer than 1% of the species within *Astragalus* (Watrous and Cane, 2011). Most species within *Astragalus* have not been reviewed since work done in the late 19th century (Bunge, 1868, 1869; Taubert, 1894).

Species of *Astragalus* can be found on every continent except Australia and Antarctica (Lewis et al., 2005). Within its nearly cosmopolitan distribution, *Astragalus* exhibits particularly rich diversity within two geographic areas. The most diverse of these

areas, home to around 2000 species, and assumed to be the origin of the genus, are the steppes and mountains of southwest and south-central Asia, and the Himalayan plateau (Wojciechowski, 2005). Second only to its likely place of origin in Eurasia, with around 400-450 species, is the intermountain region of western North America (Liston, 1997). Around 70 species of *Astragalus* can be found in Idaho (Mancuso, 1999).

Sanderson and Wojciechowski (1996) determined *Astragalus* to possess a similar diversification rate to closely related taxa (*Oxytropis* and seventeen other genera in Galegeae were included in the study), yet *Astragalus* contains many more species than these other genera. Another mechanism must explain the great diversity of *Astragalus*. The notable diversity of *Astragalus* in the intermountain west in particular may be explained by adaptive radiation, a process in which a group of organisms rapidly diversifies into several new species. Among the factors driving adaptive radiation is the sudden availability of new ecological niches (Schluter, 2000). It is possible that upon colonization of North America, *Astragalus* encountered a lack of competition for niches within environments it was well-suited for, and subsequently underwent an adaptive radiation event, quickly diversifying into several new species.

Among the species of *Astragalus* in the intermountain west is *Astragalus cusickii*. First described by Gray in 1878, *A. cusickii* is a sparsely leafy, multi-stemmed, perennial forb found in western Idaho, eastern Oregon, and the extreme southeast corner of Washington. It has small flowers and conspicuous papery inflated pods. It is found mainly on barren, often steep hillsides, ash soils, and talus slopes (Barneby et al., 1989; Mancuso, 1999). The species comprises four infraspecific taxa, varieties *cusickii*, *flexilipes*, *sterilis*, and *packardiae*. The inclusion of these four taxa into a single species

was made on the basis of morphological similarity. *Astragalus cusickii* var. *cusickii* (fig. 1) has the widest geographic distribution of the four varieties, and is found in western Idaho, eastern Oregon, and southeast Washington, though it is mostly concentrated in areas near Hells canyon (fig. 2). Individuals of *A. cusickii* var. *cusickii* are generally the largest in physical size of the varieties. Notably, they possess an approximately even distribution of leaves throughout the length of the stems, in contrast to some of the other varieties (Barneby et al., 1989). The flowers are relatively larger than those of the other varieties. The pods of *A. cusickii* var. *cusickii* have a more inflated appearance and are often found in greater abundance after flowering than in the other varieties. *Astragalus cusickii* var. *flexilipes* (fig. 3) appears to be only weakly differentiated from *A. cusickii* var. *cusickii* by subtle morphological differences: small, purplish flowers, and oblique, half-ellipsoid pods (Barneby et al., 1989). It can be found in the vicinity of the Salmon river (fig. 2). *Astragalus cusickii* var. *sterilis* (fig. 4) is one of two rare varieties, found only in a small area in eastern Oregon, near the Owyhee reservoir (fig. 2). This variety is distinguished by its smaller leaflets, and bright red mottling on its pods.

Astragalus cusickii var. *packardiae* (fig. 5) is the other rare variety. *Astragalus cusickii* var. *packardiae* is considered one of the rarest plants in Idaho (Mancuso, 1999). It was discovered in Idaho in 1980 by James Grimes and Patricia Packard, and then not seen again for seventeen years, until rediscovered by Michael Mancuso in 1997 (Mancuso, 1999). This variety is distinguished by its relative paucity of leaves on the stems, particularly distally. Its flowers are relatively small, and purplish, and its pods are small and narrow. *Astragalus cusickii* var. *packardiae* exists only in a small geographic area in Payette County, Idaho (fig. 2), and is of urgent conservation concern due to its

location on public land which is a popular site for recreational off-road vehicle use. Recent work by Kinter has found that *A. cusickii* var. *packardiae* is highly dependent on pollination by native *Osmia* species, which are also susceptible to ground disturbances (L. Kinter, unpublished). Because of these conservation concerns, *A. cusickii* var. *packardiae* is the primary focus of this study.

Astragalus cusickii var. *packardiae* exists almost entirely on a visually distinct soil type which is in sharp contrast to the surrounding areas. Small exposures of this whitish substrate dot the landscape, especially on steep slopes. These exposures are sparsely covered in vegetation. Given the abrupt change in vegetation and visually distinct nature of these exposures, it is reasonable to assume that biologically significant differences exist in the edaphic properties of the exposures versus the surrounding landscape. Unique edaphic conditions often determine the narrow distribution of rare endemic plants (Kruckeberg and Rabinowitz, 1985). It is important when considering the taxonomic organization of *A. cusickii* to consider the possibility that the morphological variation observed between the different varieties may be phenotypic plasticity in response to different edaphic conditions. It is possible that the four varieties of *A. cusickii* represent a single meta-population, with gene flow across the entire distribution, in which individual populations exhibit observable phenotypic variation in response to the particular type of soil they are found on. Given their geographic proximity and morphological similarity to the other varieties of *A. cusickii*, as well as the tendency of plants to exhibit strong physiological responses to different environments (Gurevitch et al., 2002), it is possible that the distinct morphological characters apparent in *A. cusickii* vars. *packardiae* and *sterilis* are the result of their environments.

Environmental factors were found to likely be the cause of phenotypic variation between two varieties of *Eriogonum shockleyi* (Smith and Bateman, 2002). Populations of each variety were found to often contain at least one individual which would qualify as belonging to the other variety based on their diagnostic characters. Molecular analysis did not support the taxonomic arrangement of the two varieties, making it likely that the observed variation is the result of phenotypic plasticity.

Research Approach

There are multiple potential strategies for determining the source of phenotypic variation between the varieties of *Astragalus cusickii*. One approach would be to observe the phenotypic response of individual *A. cusickii* after being transplanted to different soil types in a common garden experiment (Clausen et al., 1948; Núñez-Farfán and Schlichting, 2001). While this method would directly address whether or not environmental conditions were responsible for the observed phenotypic variation between varieties, it would also present some challenges regarding the focus taxon of this study. In 1999, it was estimated that there were approximately 3500-4500 individual *A. cusickii* var. *packardiae* in total (Mancuso, 1999). Populations of the variety are the focus of conservation efforts due to their location which makes them vulnerable to various disturbances. It is therefore important that any study of *A. cusickii* var. *packardiae* harm as few individuals as possible.

If the phenotypic variation between varieties of *Astragalus cusickii* is solely the result of phenotypic plasticity in response to different environmental conditions, it would be reasonable to expect some level of gene flow between varieties. A phylogenetic analysis could determine the degree of genetic isolation and/or gene flow among the

varieties of *A. cusickii*, for the cost of only a few small leaf tissue samples, an advantage when studying rare taxa. We propose to resolve the species boundaries within and surrounding the four varieties of *A. cusickii* using molecular phylogenetic techniques, to be evaluated using the criteria of the phylogenetic species concept – monophyly with diagnosable differences – within a larger understanding of species as separately evolving meta-population lineages, as described in the unified species concept. An advantage of using monophyly with diagnosable differences as the *a priori* standard for determining species status is that, among the various other criteria included under the umbrella of the unified species concept, it most directly attempts to elucidate the historical genealogical relationships among taxa. This is obviously of particular interest to the field of systematics. Recent studies of species in *Astragalus* using similar methods add further support to the rationale to proceed with monophyly with diagnosable differences between populations as the criteria for recognizing species (Riahi et al., 2011; Scherson et al., 2005; Wojciechowski, 2005). For the varieties of *A. cusickii*, establishing monophyly would implicitly lead to recognition as species, as the morphology of these taxa have been thoroughly studied and described, and diagnosable differences already established (Barneby et al., 1989; Mancuso, 1999). If *A. cusickii* var. *packardiae* is found to not represent an independent evolutionary lineage, but is instead simply the phenotypic result of the much more common *A. cusickii* growing in a distinct soil type, then the urgency of protecting populations of *A. cusickii* var. *packardiae* diminishes considerably. If, however, *A. cusickii* var. *packardiae* is found to represent a distinct evolutionary lineage, it would merit recognition as a species under the phylogenetic species concept. The case for conservation would therefore be bolstered.

MATERIALS AND METHODS

Collection and DNA Extraction

Sequences included in this study originated from GenBank and DNA extracted from field-collected individuals (Appendix A). Field-collected individuals were gathered from southwestern Idaho and eastern Oregon (fig. 2). Leaf material was collected from 20 individuals from five populations of *Astragalus cusickii* var. *packardiae*, nine individuals from three populations of *A. cusickii* var. *sterilis*, twelve individuals from four populations of *A. cusickii* var. *cusickii*, and nine individuals from three populations of *A. cusickii* var. *flexilipes*. In addition to the four varieties of *A. cusickii*, seven additional species of *Astragalus* were collected and included in the study on the basis of similar leaf and/or fruit morphology to *A. cusickii*. These seven species are: *A. whitneyi* var. *confusus, A. solitarius, A. lentiginosus, A. filipes, A. mulfordiae, A. yoder-williamsii,* and *A. ceramicus*. *Astragalus purshii*, which does not exhibit similar leaf and/or fruit morphology to *A. cusickii*, was also included in the study. Leaf material was preserved in silica gel. DNA was extracted from frozen and pulverized leaf tissue with Qiagen DNeasy plant mini kits (Valencia, CA) according to manufacturer's instructions.

PCR and Investigation of Gene Regions

Fifteen gene regions were chosen for investigation based on success in previous molecular systematics studies and their potential utility for species-level resolution (table 1). Low-copy nuclear, nuclear ribosomal, and chloroplast gene regions were targeted to include a broad survey of the genome, a necessary approach due to a lack of species-level

resolution in previous studies (Riahi et al., 2011; Scherson et al., 2005; Wojciechowski, 2005).

Four regions have been used in previous phylogenetic analyses of *Astragalus*. The internal transcribed spacer (*ITS*) region was investigated using the *C26A* and *NC18s10* primers (Wen and Zimmer, 1996). The utility of *ITS* for systematic studies of plants was recognized by Baldwin (1992) and has been important in phylogenetic studies at the species level in many groups, including: Rosaceae (Lee and Wen, 2001), Betulaceae (Whitcher and Wen, 2001), Rutaceae (Navarro et al., 2004), Apiaceae (Spalik and Downie, 2006, 2007; Zhou et al., 2009; Carlson et al., 2011), Lamiaceae (Oliveira et al., 2007), Piperaceae (Smith et al., 2008; Jaramillo et al., 2008), Crassulaceae (Carillo-Reyes et al., 2008), Saxifragaceae (Xiang et al., 2012), Gesneriaceae (Smith et al., 2013), and Papaveraceae (Pérez-Gutiérrez et al., 2015). The external transcribed spacer (*ETS*) region was initially developed for phylogenetic analyses by Baldwin and Markos (1998) and investigated here using the primers developed by Riahi et al. (2011) who used them in *Astragalus*. The cyclic nucleotide-gated channel 4 (*CNGC4*) protein-coding gene was developed by Choi et al. (2004, 2006) and its utility phylogenetic studies of *Astragalus* was demonstrated by Scherson et al. (2005). Scherson et al. (2005) used this low copy nuclear gene in a phylogenetic analysis of *Astragalus* and it has also been used in the Fabaceae tribe Amorpheae (McMahon, 2005). The *trnS-G* gene region was developed by Shaw et al. (2005). Riahi et al. (2011) successfully used this chloroplast spacer in a phylogenetic analysis of *Astragalus*, and it has been used in studies of other taxa in Fabaceae (Zhang et al., 2009). Therefore these four regions were included in this investigation.

Unfortunately, resolution and support has been poor using the four previously mentioned regions, albeit all four have not been used in concert prior to this study. Therefore additional regions that have been used at the species level in phylogenetic analyses were also investigated. An additional four chloroplast regions were sampled. The *trnD-T* spacer (Demesure et al., 1995) had also been used in phylogenetic studies of *Astragalus* (Scherson et al., 2008) although the level of variation was low. The *psbA-trnH* spacer (Shaw et al., 2005) has also been used in phylogenetic studies of *Astragalus* in several studies (Zippel and Wilhalm, 2009; Javanmardi et al., 2012; Bartha et al., 2013; Dastpak et al., 2013), and was thought to be an important locus to include. The *matK* gene (Sang et al., 1997) had also been used in phylogenetic analyses of *Astragalus* (Wojciechowski, 2005; Javanmardi et al., 2012), and in other taxa of Fabaceae (Miller and Bayer, 2001, 2003; Miller et al., 2003). The chloroplast encoded, nuclear expressed glutamine synthetase gene (Emshwiller and Doyle, 1999) had not been used previously in *Astragalus*, but part of this region has been used in phylogenetic studies of Gesneriaceae (Smith et al., 2004; Perret et al., 2003) and was thought to be potentially useful for this project.

The remaining seven regions were all low copy nuclear genes. The chalcone synthase, calmodulin, and glyceraldehyde phosphate dehydrogenase genes (Strand et al. 1997) have been used in several studies at the species level including: Brassicaeae (Koch et al., 2000), animals (Duda et al., 2001), fungi (O'Donnell et al., 2000; Wang and Zhuang, 2007; Romeo et al., 2011), and Piperaceae (Smith et al., 2008). The granule-bound starch synthase (*waxy*) gene was investigated using primers developed by Mason-Gamer (2001) and has been used in phylogenetic studies including Solanaceae and

Orobanchaceae (Peralta and Spooner, 2001; Tank and Olmstead, 2009). Sequences of phosphoenolpyruvate carboxylase (PEPC; Malcomber 2002) have had less use in phylogenetic analyses (Gehrig et al., 2001; Lohmann, 2006; Mason-Gamer et al, 2010), but preliminary data from other studies have shown considerable variation for this region (J. F. Smith, pers. comm.). The *ARG-10* and *FENR* genes Choi et al. (2004) was partly investigated in *Astragalus* by Scherson et al. (2005) although they detected what appeared to be multiple copies of these two regions and recommended that cloning explore the potential for paralogs.

DNA was amplified by polymerase chain-reaction (PCR) using the methods of Smith et al. (1997). Sequences were obtained from Genewiz (South Plainfield, NJ). Examination of direct sequencing products of *FENR* and *ARG-10* showed multiple peaks in their chromatograms, indicating that the primers might be amplifying multiple paralogs. To isolate orthologous genes, molecular cloning was used. Cloning was conducted with the pGEM-T vector kit from Promega (Madison, WI). Cells were plated onto luria broth agar plates. Agar plates contained 100 mg/ml ampicillin and were treated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (x-gal) and isopropyl β-D-1-thiogalactopyranoside (IPTG). For both genes, cloning was employed using five individuals: *Astragalus cusickii* var. *packardiae* JZ-002, *A. cusickii* var. *sterilis* JZ-015, *A. solitarius* JZ-021, *A. lentiginosus* JZ-036, and *A. whitneyi* var. *confusus* JZ-061. Plates were incubated overnight at 37°C. White colonies were used as templates for subsequent PCR amplification for each gene region. For *FENR*, DNA from a total of 35 colonies among the five individuals was sequenced. For *ARG-10*, DNA from a total of 50 colonies among the five individuals was sequenced.

Matrix Assembly

Sequence data from all individuals of *Astragalus cusickii* var. *packardiae* were included in the analysis, as this taxon is the focus of the study. Field-collected individuals from the other varieties of *A. cusickii* and other species of *Astragalus* were represented by a single individual from each population from which they were gathered. GenBank sequence data from seventeen more *Astragalus* species from western North America were added to supplement the collected individuals: *A. allochrous*, *A. arizonicus*, *A. asymmetricus*, *A. asclepiadoides*, *A. brandegeei*, *A. calycosus*, *A. douglasii*, *A. falcatus*, *A. inyoensis*, *A. lonchocarpus*, *A. mollissimus*, *A. nothoxys*, *A. oxyphysus*, *A. pachypus*, *A. preussii*, *A. tetrapterus*, and *A. woodruffii* (Appendix A). To attempt to place *A. cusickii* and the other western North American *Astragalus* species in a larger phylogenetic context, GenBank sequences from species occurring in other geographic areas were added. These include four *Astragalus* species native to North America, but which are not exclusively found in the west: *A. adsurgens*, *A. alpinus*, *A. americanus*, and *A. canadensis*, (Appendix A). Seventeen *Astragalus* species native to South America were included: *A. arnottianus*, *A. amatus*, *A. berteroanus*, *A. cruckshanksii*, *A. cryptobotrys*, *A. curvicaulis*, *A. cysticalyx*, *A. darumbium*, *A. edmonstonei*, *A. johnstonii*, *A. looseri*, *A. monticola*, *A. nivicola*, *A. patagonicus*, *A. pehuenches*, *A. uniflorus*, and *A. vagus* (Appendix A). Seven old world *Astragalus* species were included: *A. atropilosulus*, *A. cerasocrenus*, *A. complanatus*, *A. corrugatus*, *A. epiglottis*, *A. peristereus*, and *A. vogelii*. *Oxytropis sericea* was included as the out-group (Scherson et al., 2005).

Sequence data were manually aligned and edited for quality using PhyDE (Müller at al., 2010). There was a 30 base pair reverse complement of a section of *trnS-G* in some

individuals. The sequence was manually reversed for the individuals that had the minority version, to match the majority. The presence of the majority or minority version of this sequence was coded as a single character state appended to the sequence data for each individual in the maximum parsimony (MP) analyses.

Sequence data were not available for all individuals and all gene regions. Three concatenated super-gene matrices were assembled to determine the extent to which the missing data would affect phylogenetic analyses. A preliminary maximum parsimony analysis of individual gene regions was undertaken prior to concatenation. An incongruence was detected in the placement of three individuals of *A. cusickii* var. *packardiae* (JZ-052, JZ-053, and JZ-054) (figs. 6, 7). These individuals were removed from all gene matrices. Matrix 1 contained all available data for all remaining taxa. Matrix 1 included *Oxytropis sericea* as the out-group. Matrix 2 contained individuals for which sequence data from at least two gene regions were available. Matrix 2 also included *Oxytropis sericea* as the out-group. Matrix 3 contained only individuals for which sequence data was available for *ITS, ETS, CNGC4,* and *trnS-G*. Consequently, matrix 3 does not contain any sequences from GenBank, including the out-group *Oxytropis sericea*, as sequence data from GenBank was only available for, at most, two of the gene regions examined. For this reason, matrix 3 used *Astragalus purshii* as the out-group in place of *Oxytropis sericea*, based on the phylogenetic position of *A. purshii* in preliminary analysis of all data.

Phylogenetic Analyses

Simple indel coding was conducted with the SeqState plug-in for PhyDE. Maximum parsimony analysis was conducted on all three matrices both with and without

indel coding, using TNT version 1.1 (Goloboff et al., 2008). Default settings were used unless otherwise specified. A strict consensus tree was generated with TNT. Branch support values for the strict consensus tree were found with bootstrapping (Felsenstein, 1985) for 10,000 replicates using a tree bisection reconnection swapping algorithm. Branch arrangements were considered significant for bootstrap values ≥ 75. Matrix 1 was chosen as the dataset to proceed with all subsequent analyses (see results).

Maximum likelihood (ML) was tested using RAxML-HPC2 (Stamatakis, 2014) on XSEDE on the Cipres Science Gateway (Miller et al., 2010). *Oxytropis sericea* was specified as the outgroup. One hundred bootstrap iterations were used. Branch arrangements were considered significant where bootstrap values ≥ 75. The GTRCAT model was used. The dataset was not partitioned. FigTree v. 1.4.2 (Rambaut, 2006) was used to visualize the best tree with bootstrap values.

Prior to the Bayesian inference (BI) analysis, appropriate partitions were found using PartitionFinder (Lanfear et al., 2012). Nucleotide substitution models for each partition were found using jModelTest 2.1 (Darriba et al., 2012). Bayesian inference was tested using MrBayes 3.2.3 (Altekar et al., 2004; Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) on XSEDE on the Cipres Science Gateway (Miller et al., 2010). Two independent analyses were conducted using four Metropolis-coupled Markov chains (MCMC) (Geyer, 1991; Hastings, 1970; Metropolis et al., 1953) each for ten million generations. Burn-in was set at 50,000 generations. Metropolis-coupled Markov chain analysis completion was tested with AWTY (Nylander et al., 2007) and Tracer v. 1.6 (Rambaut et al., 2014). FigTree v. 1.4.2 (Rambaut, 2006) was used to

visualize the majority-rule consensus tree with branch posterior probabilities. Branch arrangements were considered significant where posterior probability values ≥ 0.95.

Testing Alternative Topologies

The phylogenetic analyses did not recover the four varieties of *Astragalus cusickii* as a single monophyletic group. To further investigate the potential that they represent a monophyletic group, an approximately unbiased (AU) test (Shimodaira, 2002) of the four varieties of *A. cusickii* was performed with CONSEL (Shimodaira and Hasegawa, 2001). Two additional AU tests were conducted to investigate the relative power of the AU test as applied to *Astragalus* at the species level. In these additional tests the four varieties of *A. cusickii* were specified as belonging to a monophyletic group with a morphologically similar species (*A. whitneyi* var. *confusus*) and, separately, with a much less morphologically similar species (*A. solitarius*). Site likelihoods generated in PAUP* were input into CONSEL. The significance level was designated $\alpha = 0.05$ (Lang et al., 2002; Dantrakool et al., 2004; Shannon et al., 2005; Gill and Fast, 2006; Heiss and Keeling 2006; Struck et al., 2007; Ernst et al., 2008; Helmkampf et al., 2008; Kuo et al., 2008; Yu et al., 2008; Gao et al., 2009; Ishiwata et al., 2011; Zhang et al., 2011).

The genealogical sorting index (GSI) (Cummings et al., 2007) was used to further investigate the possibility of a monophyletic *Astragalus cusickii*. A file containing the last 100 trees (the maximum number allowed by the GSI software) generated in the BI analysis, as well as text files used to specify monophyletic constraints among taxa, were used as the inputs. The significance level was designated at $\alpha = 0.05$ (Koopman and Baum, 2010; Kubatko et al., 2011; Keith and Hedin, 2012; Levsen et al., 2012).

The following monophyletic arrangements were defined and tested against the BI data using the GSI (table 2): 1. each variety of *Astragalus cusickii* as independent monophyletic groups, 2. all four varieties of *A. cusickii* as a single monophyletic group, 3. *A. cusickii* as three separate monophyletic groups: *A. cusickii* var. *packardiae, A. cusickii* var. *sterilis,* and a combined group containing *A. cusickii* vars. *cusickii* and *flexilipes*, 4. all four varieties of *A. cusickii* combined with *A. whitneyi* var. *confusus* as a monophyletic group, and 5. all four varieties of *A. cusickii* combined with *A. solitarius* as a monophyletic group. The rationale for combining *A. cusickii* vars. *cusickii* and *flexilipes* was based on the results of the phylogenetic analyses and their status as weakly differentiated varieties (Barneby, 1989). *Astragalus whitneyi*, a morphologically similar species to *A. cusickii*, and *A. solitarius*, a less morphologically similar species to *A. cusickii*, were included in separate analyses as a test of the relative power of the GSI as applied to *Astragalus* at the species level.

Multispecies Coalescent

Species trees were estimated from sequence data from individuals belonging to species for which more than one individual was included in the study, and for which sequence data was available for *ITS, ETS, CNGC4,* and *trnS-G*, using the multispecies coalescent (Kingman, 1982, 2000; Hudson, 1991; Knowles and Carstens, 2007; Degnan and Rosenberg, 2009; Heled and Drummond, 2010; Carstens et al., 2013). The *BEAST template (Drummand et al., 2012) was used in BEAUti v. 2 to prepare the data file for multispecies coalescent analysis in BEAST v. 2.1.3 (Bouckaert et al., 2014). Separate nexus files for each partition suggested by PartitionFinder were used as inputs for *BEAST. Models were unlinked among partitions. Two independent analyses using each

of the four available substitution models (JC69, HKY, TN93, GTR), separately using a strict clock or a relaxed log normal clock were conducted for 100 million generations each, for a total of sixteen analyses. All other parameters were left at default settings. Additionally, two independent analyses using substitution model JC69 with a strict clock were conducted for one billion generations each. Tracer v. 1.6.0 (Rambaut et al., 2014) was used to gauge MCMC convergence. TreeAnnotator v. 2.1.2 (Rambaut and Drummond, 2002), an application in *BEAST, was used to generate target trees for each analysis under the maximum clade credibility criterion. FigTree v. 1.4.2 (Rambaut, 2006) was used to visualize the trees generated by TreeAnnotator. *BEAST results were considered supported at posterior probability > 0.95 (Niemiller et al., 2012; Perez et al., 2012; Kearns et al., 2013; Satler et al., 2013).

RESULTS

Amplification, Sequencing, and Alignment

The internal transcribed spacer (*ITS*) gene region was successfully amplified and sequenced for all individuals from which DNA was extracted (table 1; Appendix A). The *ITS* sequences were available for all 46 individuals accessed from GenBank. An average proportion of nucleotide differences between individuals (pairwise distance) of 0.034 was calculated. The level of variation found among *ITS* sequences was sufficient to generate informative topology from the maximum parsimony analysis (fig. 6), including BS support of 85 for a monophyletic *Astragalus cusickii* var. *packardiae*.

The *trnS-G* chloroplast gene region was successfully amplified and sequenced for all individuals from which DNA was extracted (table 1; Appendix A). No *trnS-G* sequences were available for taxa accessed from GenBank. An average pairwise distance of 0.006 was calculated. The level of variation found among *trnS-G* sequences was sufficient to generate informative topology from the maximum parsimony analysis (fig. 7), including BS support of 91 for a monophyletic *Astragalus cusickii* var. *sterilis*. A bootstrap-supported incongruence was found between the *trnS-G* (plastid) gene tree (fig. 7) and the *ITS* gene tree (fig. 6). The incongruence occurred in the placement of individuals from one population of *A. cusickii* var. *packardiae* (JZ-052, JZ-053, and JZ-054) in a clade containing an individual of *A. filipes* (DM 13-005) with branch support of 87 (fig. 7).

The external transcribed spacer (*ETS*) gene region was successfully amplified and sequenced for 53% of individuals from which DNA was extracted (table 1; Appendix A). No *ETS* sequences were available from GenBank for the additional taxa included. An average pairwise distance of 0.015 was calculated. The cyclic nucleotide-gated channel 4 (CNGC4) gene was successfully amplified and sequenced for 98% of the individuals from which DNA was extracted (table 1; Appendix A). Cyclic nucleotide-gated channel 4 sequences were available from GenBank for 26% of individuals accessed from GenBank. An average pairwise distance of 0.014 was calculated.

The *trnD-T* and *matK* gene regions were successfully amplified and sequenced for 53% and 84% of individuals from which DNA was extracted, respectively. Sequence alignment for both regions showed negligible variation between taxa. Two gene regions, *FENR* and *ARG10*, showed evidence of multiple paralogs, which were explored through cloning. After cloning, seven paralogs were detected for *FENR*, and eight for *ARG10*, within a single individual of *Astragalus solitarius* (JZ-021), with similar numbers of paralogs found among other individuals. As a result, sequence variation for *FENR* and *ARG10* was greater between paralogs than between taxa. For this reason, these gene regions were not included. Amplification was not successful for the remaining seven gene regions (table 1).

Phylogenetic Analyses

Maximum parsimony analysis of matrix 1 (fig. 8), containing sequence data from 89 individuals, resulted in 30 equally most-parsimonious trees (L = 689, CI = 0.805, RI = 0.869). There was strong support for a monophyletic *Astragalus cusickii* var. *packardiae* (BS = 95) and a monophyletic *A. cusickii* var. *sterilis* (BS = 84). *Astragalus cusickii* var.

cusickii and *A. cusickii* var. *flexilipes* did not resolve as reciprocally monophyletic, and instead formed a combined clade lacking bootstrap support.

Maximum parsimony analysis of matrix 2 (fig. 9), containing sequence data from 55 individuals, resulted in 20 equally most-parsimonious trees (L = 419, CI = 0.886, RI = 0.929). In general, the results are similar to those found in the analysis of matrix 1: strong bootstrap support for a monophyletic *A. cusickii* var. *packardiae* (BS = 97) and a monophyletic *A. cusickii* var. *sterilis* (BS = 96), as well as an unsupported combined clade including *A. cusickii* var. *cusickii* and *A. cusickii* var. *flexilipes*.

Maximum parsimony analysis of matrix 3 (fig. 10), containing sequence data from 24 individuals, resulted in a single most-parsimonious tree (L = 154, CI = 0.883, RI = 0.941). Matrix 3 showed high bootstrap support for the reciprocal monophyly of three of the varieties of *Astragalus cusickii*: *A. cusickii* var. *packardiae* (BS = 100), *A. cusickii* var. *sterilis* (BS = 99), and *A. cusickii* var. *cusickii* (BS = 92). One variety, *A. cusickii* var. *flexilipes*, was not included in matrix 3 as sequence data were not available for all gene regions.

Comparison of the three matrices shows a trend toward less support as additional taxa lacking sequence data from one or more gene region are added. The proportion of supported nodes is highest in matrix 3 (73%) and drops with additional taxa in matrices 2 and 1 (30% and 29%, respectively) (fig. 8, 9, 10). Matrix 1 was chosen as the data set for use in all subsequent analyses, because although it has the lowest proportion of supported nodes, it included the most taxa, thereby maximizing the phylogenetic space available to resolve relationships within *A. cusickii*.

The results from the ML analysis were in agreement with the results from the MP analysis (fig. 11). *Astragalus cusickii* var. *packardiae* and *A. cusickii* var. *sterilis* each received significant bootstrap support as reciprocally monophyletic groups (96 and 100, respectively). *Astragalus cusickii* var. *cusickii* and *A. cusickii* var. *flexilipes* form an unsupported combined clade. Branch support for many of the clades was greater in the ML analysis, in contrast to the MP analysis, though in most cases still below the level of significance of 75.

PartitionFinder indicated that each of the four gene regions should be partitioned, as well as each codon position in *CNGC4* (a protein-coding gene region), for a total of six partitions. Each partition was assigned a unique model by jModelTest, with the exception of *CNGC4* codon positions one and two, which were assigned the same model (table 3). The MCMC trace plot did not have an apparent vertical trend (fig. 12), suggesting MCMC completion. A joint-marginal plot of two independent BI analyses (fig. 13) is consistent with MCMC convergence. Metropolis-coupled Markov chain completion was supported by AWTY (fig. 14). The first of the independent analyses had a posterior mean of -5520.91, and a posterior effective sample size (ESS) of 3373. The second independent analysis had a posterior mean of -5520.74, and a posterior ESS of 3015. The BI analysis produced results in agreement with the MP and ML analyses (fig. 11). *Astragalus cusickii* var. *packardiae* and *A. cusickii* var. *sterilis* each received strong posterior probabilities (1.00 and 1.00, respectively) as comprising reciprocally monophyletic groups. *Astragalus cusickii* var. *cusickii* and *A. cusickii* var. *flexilipes* form a combined clade with PP = 0.922. In general, branch support for many of the clades was greatest in the BI analysis of the three phylogenetic analyses employed.

Tests of Alternative Topologies

The AU test could not reject the possibility of a monophyletic *Astragalus cusickii* containing all varieties (p = 0.911). The AU test (table 4) also failed to reject the possibility of a monophyletic combination of *A. cusickii* and *A. whitneyi* var. *confusus* (p = 0.746), but did reject a monophyletic combination of *A. cusickii* and *A. solitarius* (p = 0.005).

The GSI supported separately monophyletic *Astragalus cusickii* var. *packardiae* and *A. cusickii* var. *sterilis* (table 2). Both taxa received the maximum score of 1.00 from the GSI when each was constrained to be monophyletic, indicating complete lineage sorting had occurred. *Astragalus cusickii* var. *cusickii* and *A. cusickii* var. *flexilipes* were supported as comprising a single clade in the results of the GSI (score of 0.989). When evaluated separately, *A. cusickii* var. *cusickii* received a GSI score of 0.796, and *A. cusickii* var. *flexilipes* received a score of 0.442. Evaluated as a single group, the four varieties of *A. cusickii* received a GSI score of 0.874. Including *A. whitneyi* var. *confusus* returned a GSI score of 0.874, including *A. solitarius* with the varieties of *A. cusickii* returned a GSI score of 0.839.

Multispecies Coalescent

Results from all multispecies coalescent analyses produced species trees with identical topology, though with different posterior probabilities. The multispecies coalescent did not support *Astragalus cusickii* as monophyletic (fig. 15). In the analysis ran for one billion generations *Astragalus cusickii* var. *cusickii* and *A. cusickii* var. *sterilis* were grouped into an unsupported clade sister to a supported clade (PP = 1.00) containing *A. purshii* and *A. lentiginosus*. *Astragalus cusickii* var. *packardiae* was placed as the

outgroup to the other taxa, though this arrangement was without support. Effective sample sizes for all analyses are reported in table 5. Joint-marginal distributions indicate a lack of convergence between parallel analyses (fig. 16).

DISCUSSION

In general, the results of all three phylogenetic methods produced similar to identical results which were also reflected with the MP analyses of data matrices with reduced taxon sampling, but complete sequence data for all sampled individuals (figs. 8-11). The results provide strong support for some clades, typically clusters of individuals where more than a single individual was sampled, but poor support for relationships among species. Phenotypic variation found in *Astragalus cusickii* vars. *packardiae* and *sterilis* in relation to the other varieties of *A. cusickii* appears to be genetically determined. The MP, ML, and BI analyses each strongly support a monophyletic *A. cusickii* var. *packardiae*, and a monophyletic *A. cusickii* var. *sterilis* (fig. 11). A clade exclusively containing all individuals of *A. cusickii* var. *packardiae* received the maximum possible posterior probability of 1.000 in the BI analysis, and similarly robust support in the other phylogenetic analyses (fig. 11). The clade containing *A. cusickii* var. *sterilis* was similarly well-supported, receiving a posterior probability of 1.000 in the BI analysis, and 100 in the ML analysis (fig. 11). Such clearly defined clades are likely the result of long-term isolation, resulting in the accumulation of unique genetic mutations.

Phenotypic plasticity is a possible explanation for the subtle phenotypic variation between *Astragalus cusickii* vars. *cusickii* and *flexilipes*. These two varieties exist in overlapping territory (fig. 2), and are considered weakly differentiated (Barneby et al., 1989). Neither variety resolved as monophyletic in the phylogenetic analyses. *Astragalus*

cusickii vars. *cusickii* and *flexilipes* formed a combined, though unsupported, monophyletic clade in each phylogenetic analysis (fig. 11).

Tests of Alternative Topologies

The phylogenetic analyses did not support a monophyletic *Astragalus cusickii* containing all varieties, but also did not preclude such a group. Alternative tests of topology were employed in an attempt to recover relationships, which may have been missed by the traditional analyses. The approximately unbiased (AU) test (Shimodaira, 2002) calculates a p-value for a user-defined monophyletic group given a particular dataset. The AU has been used in a broad range of phylogenetic studies, including taxa such as protists (Lang et al., 2002; Kuo et al., 2008), rats (Dantrakool et al., 2004), nematodes (Shannon et al., 2005), leeches (Trontelj and Utevsky, 2005), octocorals (Wirshing et al., 2005), flagellates (Heiss and Keeling, 2006), fish (Steinke et al., 2006; He and Chen, 2006; Willis et al., 2012), fungi (Gill and Fast, 2006; James et al., 2006), annelids (Struck et al., 2007), frogs (Ernst et al., 2008; Yu et al., 2008), plankton (Helmkampf et al., 2008), algae (Hall et al., 2008; Rindi et al., 2009; Pröschold et al., 2010), ciliates (Gao et al., 2009; Zhang et al., 2011), rosids (Wang et al., 2009), metazoans (Witek et al., 2009), crocodiles (Oaks 2011), and insects (Ishiwata et al., 2011).

The AU test failed to reject a monophyletic *Astragalus cusickii* (table 4). While the AU test could not reject a monophyletic *A. cusickii*, it also could not reject a monophyletic group containing both *A. cusickii* and *A. whitneyi* var. *confusus* (table 4). One interpretation of these data is that *A. cusickii* could be redefined to include *A. whitneyi* var. *confusus*, and potentially other taxa within the polytomy containing *A.*

cusickii (fig. 11) that were not tested here. However, monophyly alone does not imply species status. Combining what are currently thought of as separate species (*A. cusickii* and *A. whitneyi* var. *confusus*), with clear morphological differences (Barneby et al., 1989) on the basis of monophyly alone makes little sense. The phylogenetic species concept defines species not just as monophyletic groups, but the smallest such groups that are distinguishable from other groups. The results of the AU test are not inconsistent with the results of the traditional phylogenetic analyses: *A. cusickii* vars. *packardiae* and *sterilis* form monophyletic groups which may be nested within a larger monophyletic group containing the other varieties of *A. cusickii*, and perhaps *A. whitneyi* var. *confusus* as well.

The genealogical sorting index (GSI) (Cummings et al., 2007) was used to further investigate the possibility of a monophyletic *Astragalus cusickii*. The GSI calculates the degree of genealogical clustering among a set of sequences. The GSI was used to compare the support among four different groups of taxa constrained as monophyletic. Support for a particular specified monophyletic arrangement is calculated based on how such an arrangement agrees with the available data. The GSI has been used in a broad range of phylogenetic studies, including taxa such as *Oryza* (Cranston et al., 2009), Malvaceae (Koopman and Baum, 2010), harvestmen (Derkarabetian et al., 2011), fungi (Gazis et al., 2011; Sakalidis et al., 2011; Wang et al., 2014), rattlesnakes (Kubatko et al., 2011), birds (Welch et al., 2011), spiders (Keith and Hedin, 2012), *Populus* (Levsen et al., 2012), fish (Niemiller et al., 2012), lichens (Pino-Bodas et al., 2013), and *Primula* (Schmidt-Lebuhn et al., 2012). Any GSI value above zero with a corresponding p-value < 0.05 implies some degree of genealogical clustering, with a value of one indicating

completely established monophyly. No commonly used GSI value exists by which to accept a particular group of individuals as monophyletic, though values as low as 0.218 have been considered significant genealogical clustering (Koopman and Baum, 2010). Wang et al. (2014) considered GSI ≥ 0.85 to imply a "high degree of exclusive ancestry."

The GSI supported a monophyletic *Astragalus cusickii* with a value of 0.874 (table 2). However, equal support was found for a monophyletic group containing both *A. cusickii* and *A. whitneyi* var. *confusus* (GSI 0.874) (table 2). As with the AU test, these results could be interpreted as supporting the inclusion of *A. whitneyi* var. *confusus* in *A. cusickii*. However, monophyly alone does not equate to species status. The GSI provided the most robust support when considering the varieties of *A. cusickii* split into three groups: *A. cusickii* vars. *cusickii* and *flexilipes* as a combined group, and *A. cusickii* vars. *packardiae* and *sterilis* each representing separate groups (GSI 0.989, 1.000, 1.000, respectively) (table 2), consistent with the results of the traditional phylogenetic analyses. The results of the GSI demonstrate that the varieties of *A. cusickii* are at least as distinct from each other as they are from taxa that have long been considered separate species.

Multispecies Coalescent

The multispecies coalescent is an alternative approach to inferring species trees from multilocus sequence data using coalescent theory (Kingman, 1982, 2000; Hudson, 1991; Knowles and Carstens, 2007; Degnan and Rosenberg, 2009; Heled and Drummond, 2010; Carstens et al., 2013). Multilocus sequence data often leads to gene tree discordance due to incomplete lineage sorting, particularly in instances of adaptive radiation (Degnan and Rosenberg, 2009), as *Astragalus* appears to have undergone in the intermountain west. Traditional phylogenetic methods can produce inaccurate species

trees as a result (Rokas et al., 2003; Jennings and Edwards, 2005; Kubatko, 2007; Degnan and Rosenberg, 2009). The multispecies coalescent differs from traditional phylogenetic analyses, in part, in that it defines operational taxonomic units not as individuals, but as evolutionary lineages with many individuals. The calculations of the multispecies coalescent are informed by the logic that the coalescent point of two lineages of a gene from two species must occur earlier in history than the speciation event dividing the two species (Degnan and Rosenberg, 2009). The multispecies coalescent has been used in phylogenetic studies of many diverse taxa, including plants (Molina et al., 2011; Schmidt-Lebuhn et al., 2012; Zhong et al., 2013; Pillon et al., 2013; Dauphin et al., 2014; Steane et al., 2015; Stephens et al., 2015), birds (Harrington and Near, 2012; Kearns et al., 2013; Lavretsky et al., 2014), fish (Niemiller et al., 2012), mammals (Song et al., 2012; Paupério et al., 2012; Perez et al., 2012); reptiles (Spinks et al., 2012; Parham et al., 2013), mollusks (Sales et al., 2013), arachnids (Satler et al., 2013), and amphibians (Wielstra et al., 2013).

The dataset used in the multispecies coalescent analysis was significantly smaller than the dataset used in the traditional phylogenetic analyses. The multispecies coalescent requires a minimum of two individuals per species (Heled and Drummond, 2010) and the software used in the analysis requires that each included individual have sequence data corresponding to each included locus (Drummond et al., 2012). Only nineteen individuals met these requirements, and no *Astragalus cusickii* var. *flexilipes* were included (fig. 15). The analysis produced a species tree grouping *A. cusickii* var. *cusickii* and *A. cusickii* var. *sterilis* in an unsupported clade, with *A. lentiginosus* and *A. purshii*, with *A. cusickii* var. *packardiae* as sister to the other taxa (fig. 15). This is in agreement with the phylogenetic

analyses that recovered monophyletic clades for *A. cusickii* var. *packardiae* and *A. cusickii* var. *sterilis*, but did not include *A. cusickii* var. *flexilipes* or the type variety in a single monophyletic species.

Chloroplast Capture

Individuals of *Astragalus cusickii* var. *packardiae* from a single population (JZ-052, JZ-053, and JZ-054) showed signs of a chloroplast capture event not evident in other individuals. Maximum parsimony analysis of *trnS-G* sequences in isolation grouped most individuals of *A. cusickii* var. *packardiae* in a polytomy with the other varieties of *A. cusickii* and *A. whitneyi* var. *confusus* (fig. 7). Individuals from the anomalous population were placed in a supported clade (BS = 87) with one individual of *A. filipes*. These results may be explained by the phenomenon of chloroplast capture.

Chloroplast capture is a type of introgression resulting from hybridization between species. Hybridizations have occurred frequently in the evolutionary histories of many plant taxa (Ellstrand et al., 1996), but are rare within *Astragalus* (Liston, 1992; Bartha et al., 2013). Hybridization followed by backcrossing with one of the parent species will increase the proportion of that parent species' contribution to the genome of the resulting progeny. With additional generations of backcrossing, hybrid populations increasingly approach the genetic composition of one parent species, while possibly maintaining genes from the other. Over time, introgression, the transfer of genes between species, is the net result (Richards, 1986).

Due to the mechanism of inheritance of chloroplasts, hybrids formed from two species will usually possess the chloroplasts originating from only the maternal parent species (Birky, 1995), although paternal and biparental chloroplast inheritance has been

observed in Fabaceae (Corriveau and Coleman, 1988; Harris and Ingram, 1991; Rajora and Mahon, 1995), and specifically in *Astragalus* (Corriveau and Coleman, 1988; Zhang et al., 2003). If such hybrids repeatedly backcross with the parent species which did not contribute the chloroplast, their nuclear genome will increasingly resemble the nuclear genome of the parent species with which they are backcrossing, until they are indistinguishable. However, the non-recombining chloroplast genome will retain the form inherited from the parent species which contributed the chloroplast in the original hybridization event. This results in populations or species which have essentially "captured" the chloroplast of another species. Such situations give rise to discordance between the evolutionary histories of nuclear and chloroplast genes (Rieseberg et al., 1996; Tsitrone et al., 2003).

Much of the early molecular systematic work done with plants relied on the chloroplast genome to reconstruct evolutionary histories. As subsequent studies were conducted using gene regions from the nuclear genome, many of the findings of earlier studies were shown to be incorrect. Reconstructions of phylogenetic relationships at the taxonomic level of genus, species, and sub-species were especially vulnerable to the confounding effects of chloroplast capture in studies relying solely on the chloroplast genome (Soltis and Kuzoff, 1995). In one study, Soltis and Kuzoff (1995) were able to locate at least four distinct chloroplast capture events in *Heuchera*, including one likely to have occurred early in the diversification of the genus. As a result, based on earlier phylogenetic studies using only the chloroplast genome, *Heuchera* had been incorrectly thought to be closely related to *Lithophragma*, *Bensoniella*, and *Tolmiea*.

In another example from the same study (Soltis and Kuzoff, 1995), populations of *Tellima grandiflora* occurring at the southern end of their range were shown to possess a chloroplast genome closely related to that of *Mitella diversiflora* and *M. trifida*, not shared by the other populations. Sequence data from the nuclear genome, as well as morphology, chemistry, and allozyme data supported the classification of the northern and southern populations of *T. grandiflora* as a single species. A study relying on sequence data from the chloroplast genome alone would have concluded that the southern populations of *T. grandiflora* were a separate species, and that this species was more closely related to *M. diversiflora* and *M. trifida* than the northern populations of *T. grandiflora*. *Tellima grandiflora* is not known to currently form hybrids with *M. diversiflora* or *M. trifida*, indicating that the hybridization event leading to chloroplast capture occurred at some point in the past when hybridization between these taxa was more likely.

Molecular evidence indicates that a similar situation may have occurred in the anomalous population of *Astragalus cusickii* var. *packardiae*. Individuals from this population were placed into a supported clade with *A. filipes* through maximum parsimony analysis of the *trnS-G* plastid gene region (fig. 7). *Astragalus filipes* and *A. cusickii* are morphologically similar species occurring within the same general geographic area. No known hybrids between the two species have been described, but it is possible that they may have hybridized in the past. Pherograms corresponding to nuclear and nuclear-ribosomal gene regions for JZ-052, JZ-053, and JZ-054 show sharp peaks, indicating that they are homozygous at these loci, implying that hybridization was not recent.

Low Support for Clades

Beyond *Astragalus cusickii* var. *packardiae*, and *A. cusickii* var. *sterilis*, there was a general lack of support for species-level relationships across the species tree, with many of the included species grouped in polytomies (fig. 11). These results may reflect an adaptive radiation event having occurred within *Astragalus* upon colonization of the intermountain west area of North America. Species colonizing a new habitat may rapidly diversify into multiple new species to take advantage of available niches (Schluter, 2000). The high level of diversity of *Astragalus* found in the region (Liston, 1997) and the challenging nature of systematic studies of the genus (Scherson et al., 2005) are consistent with an adaptive radiation event having occurred relatively recently. The many poorly-resolved relationships found in the phylogenetic analyses may indicate that an insufficient amount of time has passed since the adaptive radiation event for complete lineage sorting to occur across the various taxa found in the region. Gene flow between some taxa may have been occurring until recently, or could still be occurring. In this context, the strong support for a monophyletic *A. cusickii* var. *packardiae* and a monophyletic *A. cusickii* var. *sterilis* could be understood to reflect a greater degree of isolation relative to other *Astragalus* species in the region, allowing for the accumulation of mutations and, eventually, synapomorphic phenotypic traits. Consistent with this hypothesis, both varieties are found in small geographic areas with non-overlapping ranges relative to the other varieties of *A. cusickii* (fig. 2).

CONCLUSION

Based on the results of the phylogenetic analyses and the genealogical sorting index, we advocate elevation of *Astragalus cusickii* var. *packardiae*, as well as *A. cusickii* var. *sterilis*, to species status. Both of these varieties were strongly supported as comprising monophyletic groups in the MP, ML, and BI analyses (fig. 11). Including loci from nuclear, ribosomal, and chloroplast DNA, which are independently inherited, results in the phylogenetic analyses having greater power to resolve species trees (Corl and Ellegren, 2013). This, considered with the outcome of the gene trees from the separate phylogenetic analyses displaying shared topology where support existed, give us confidence that the results of the phylogenetic analyses represent true monophyletic groups.

Criteria for determination of species status and boundaries were determined *a priori* to align with those of the phylogenetic species concept. Because the varieties of *Astragalus cusickii* already exhibit morphological characters which can be used to distinguish them from each other, demonstration of the monophyly of any particular variety satisfies the criteria of the phylogenetic species concept for consideration of that variety as a separate species from the other varieties of *A. cusickii*. The results of the various analyses employed in this study have demonstrated the monophyly, separately, of both *A. cusickii* var. *packardiae* and *A. cusickii* var. *sterilis*, thus showing merit for the recognition of these two taxa as species.

Recently, 160 individuals of *A. cusickii* var. *packardiae* were successfully grown in a greenhouse at the Idaho Botanical Garden (J. F. Smith, pers. comm.). These individuals were grown in a mixture of soil from their natural habitat and commercial potting soil. When grown under these conditions, individual *A. cusickii* var. *packardiae* appear to exhibit similar morphology to wild individuals, and do not show phenotypic variation similar to any of the other varieties.

The addition of two species to our description of *Astragalus* within the intermountain west expands our understanding of the extreme diversity of the genus found in the region (Liston, 1997). Across much of the genus, application of modern molecular phylogenetic analyses has not occurred. Few studies have used these modern techniques to explore the species boundaries among closely related taxa within *Astragalus* which are phenotypically similar and exist within close proximity to one another. Our appreciation of the tremendous diversity of *Astragalus* in the intermountain west is largely informed by the taxonomic work done prior to the development of the field of molecular systematics, based on morphological analysis (Bunge, 1868, 1869; Taubert, 1894). This understanding may or may not reflect an accurate estimate of the actual diversity found in the region. The results of this study demonstrate the possibility that the diversity of *Astragalus* in the intermountain west as measured by described species may be underestimated.

There is an ongoing effort to reexamine previous taxonomic work conducted primarily on the basis of morphology using modern molecular techniques. Much of the taxonomic work previously done within *Astragalus* was performed by carefully examining and comparing morphology among taxa (Hillis, 1987). It is unclear if subtle

morphological variation between populations is due to each population following its own unique evolutionary path, or simply due to environmental factors. Molecular phylogenetic studies continue to discover cryptic species within what had previously been understood to be single species (Adjie et al., 2007; Heinrichs et al., 2010; Liao et al., 2011; Carter 2012; Dong et al., 2012; Carstens and Satler, 2013; Dauphin et al., 2014).

The two rare varieties of *Astragalus cusickii, A. cusickii* var. *packardiae* and *A. cusickii* var. *sterilis*, are each following a unique evolutionary path, as demonstrated by multiple phylogenetic analyses of the genetic sequences of multiple independently inherited loci. The impetus for protecting populations of these rare species is therefore strengthened, as the extinction of either would mean the permanent loss of a unique evolutionary lineage.

TABLES

Table 1. Gene regions investigated via polymerase chain-reaction in *Astragalus* species

Gene Region	Primer Citation	Type	Result	Average Pair-Wise Distance	Matrix Length	Parsimoniously Informative
internal transcribed spacer	Wen and Zimmer, 1996	ribosomal	amplification with parsimoniously-informative variation	0.034	559 bp	87 bp
external transcribed spacer	Baldwin and Markos, 1998	ribosomal	amplification with parsimoniously-informative variation	0.015	441 bp	15 bp
cyclic nucleotide-gated channel 4	Choi et al., 2004, 2006	nuclear	amplification with parsimoniously-informative variation	0.014	396 bp	33 bp
trnS-G	Shaw et al., 2005	plastid	amplification with parsimoniously-informative variation	0.006	562 bp	71 bp
trnD-T	Demesure et al., 1995	plastid	amplification with negligible variation	n/a	n/a	n/a
matK	Sang et al., 1997	plastid	amplification with negligible variation	n/a	n/a	n/a
PEPCX4F-PEPCX5R	Malcomber, 2002	nuclear	no amplification	n/a	n/a	n/a
GPDX7F-GPDX9R	Strand et al., 1997	nuclear	no amplification	n/a	n/a	n/a
GS687f-GS856r	Emshwiller and Doyle, 1999	nuclear	no amplification	n/a	n/a	n/a
CHSX1F-CHSX2RN	Strand et al., 1997	nuclear	no amplification	n/a	n/a	n/a
CAMX1F-CAMX2R	Strand et al., 1997	nuclear	no amplification	n/a	n/a	n/a
waxy136F-waxy1699R	Mason-Gamer, 2001	nuclear	no amplification	n/a	n/a	n/a
psbAF-trnHR	Shaw et al., 2005	plastid	no amplification	n/a	n/a	n/a
FENR	Choi et al., 2004	nuclear	cloned, multiple paralogs	n/a	n/a	n/a
ARG10	Choi et al., 2004	nuclear	cloned, multiple paralogs	n/a	n/a	n/a

Table 2. Genealogical sorting index scores and corresponding p-values. P-value < 0.05 results in rejection of null hypothesis that the defined monophyletic group is incorrect. Genealogical sorting index possible scores range from 0 to 1, with 1 indicating complete lineage sorting, and 0 indicating no lineage sorting.

Monophyletic Group	GSI Score	P-value
A. cusickii var. *cusickii*	0.796	0.0001
A. cusickii var. *flexilipes*	0.442	0.001
A. cusickii var. *packardiae*	1.000	0.0001
A. cusickii var. *sterilis*	1.000	0.0001
A. cusickii var. *cusickii* + *A. cusickii* var. *flexilipes*	0.989	0.0001
A. cusickii all varieties	0.874	0.0001
A. cusickii all varieties + *A. whitneyi* var. *confusus*	0.874	0.0001
A. cusickii all varieties + *A. solitarius*	0.839	0.0001

Table 3. Models and parameters suggested by jModelTest for gene regions included in the analysis

Gene Region	model	partition	-lnL	K	freq. A	freq. C	freq. G	freq. T	R(a) [AC]	R(b) [AG]	R(c) [AT]	R(d) [CG]	R(e) [CT]	R(f) [GT]	p-inv	gamma shape	kappa	ti/tv
ITS	SYM + I + G	012345	2538.8408	183	n/a	n/a	n/a	n/a	1.1813	5.5134	2.4867	0.8975	3.7307	1.0000	0.3120	0.8120	n/a	n/a
ETS	TIM3	012032	761.6996	50	0.3675	0.2880	0.2221	0.1223	0.2299	1.0659	1.0000	0.2299	0.2258	1.0000	n/a	n/a	n/a	n/a
CNGC4 codon pos. 1	TPM3uf	012012	342.7643	181	0.3260	0.1067	0.1962	0.3711	2.3301	2.5549	1.0000	2.3301	2.5549	1.0000	n/a	n/a	n/a	n/a
CNGC4 codon pos. 2	TPM3uf	012012	307.6324	181	0.3547	0.1727	0.2171	0.2554	0.0000	2.6512	1.0000	0.0000	2.6512	1.0000	n/a	n/a	n/a	n/a
CNGC4 codon pos. 3	HKY	010010	283.1729	180	0.3734	0.2066	0.1496	0.2703	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	8.1004	3.6279
trnS-G	TIM2 + I	010232	838.2825	91	0.3907	0.1318	0.1320	0.3455	0.2564	0.1895	0.2564	1.0000	1.8973	1.0000	0.9200	n/a	n/a	n/a

Table 4. P-values resulting from the approximately unbiased test of three monophyletic arrangement hypotheses. P-value < 0.05 results in rejection of hypothesis.

monophyletic group	AU p-value
all varieties of *A. cusickii*	0.911
all varieties of *A. cusickii* + *A. whitneyi* var. *confusus*	0.746
all varieties of *A. cusickii* + *A. solitarius*	0.005

Table 5. Substitution and clock models, and resulting posterior statistics of multiple *BEAST analyses

Generations	Substitution Model	Clock Model	Posterior Mean	Posterior Effective Sample Size
100 million	GTR	relaxed	-2445.08	204
100 million	GTR	relaxed	-2421.41	11
100 million	GTR	strict	-2439.27	78
100 million	GTR	strict	-2457.38	100
100 million	HKY	relaxed	-2423.91	892
100 million	HKY	relaxed	-2426.20	1204
100 million	HKY	strict	-2434.83	1563
100 million	HKY	strict	-2434.04	1753
100 million	JC69	relaxed	-2528.09	968
100 million	JC69	relaxed	-3157.11	1636
100 million	JC69	strict	-2537.85	1683
100 million	JC69	strict	-2536.63	1535
100 million	TN93	relaxed	-2429.14	1072
100 million	TN93	relaxed	-2431.00	1259
100 million	TN93	strict	-2443.67	1606
100 million	TN93	strict	-2442.32	1480
1 billion	JC69	strict	-2537.22	8383
1 billion	JC69	strict	-2536.74	8185

FIGURES

Fig. 1. Individual *Astragalus cusickii* var. *cusickii* photographed on 27 June 2013 on a steep, gravelly slope in Hells Canyon, Adams county, Idaho. Numerous inflated papery pods are evident.

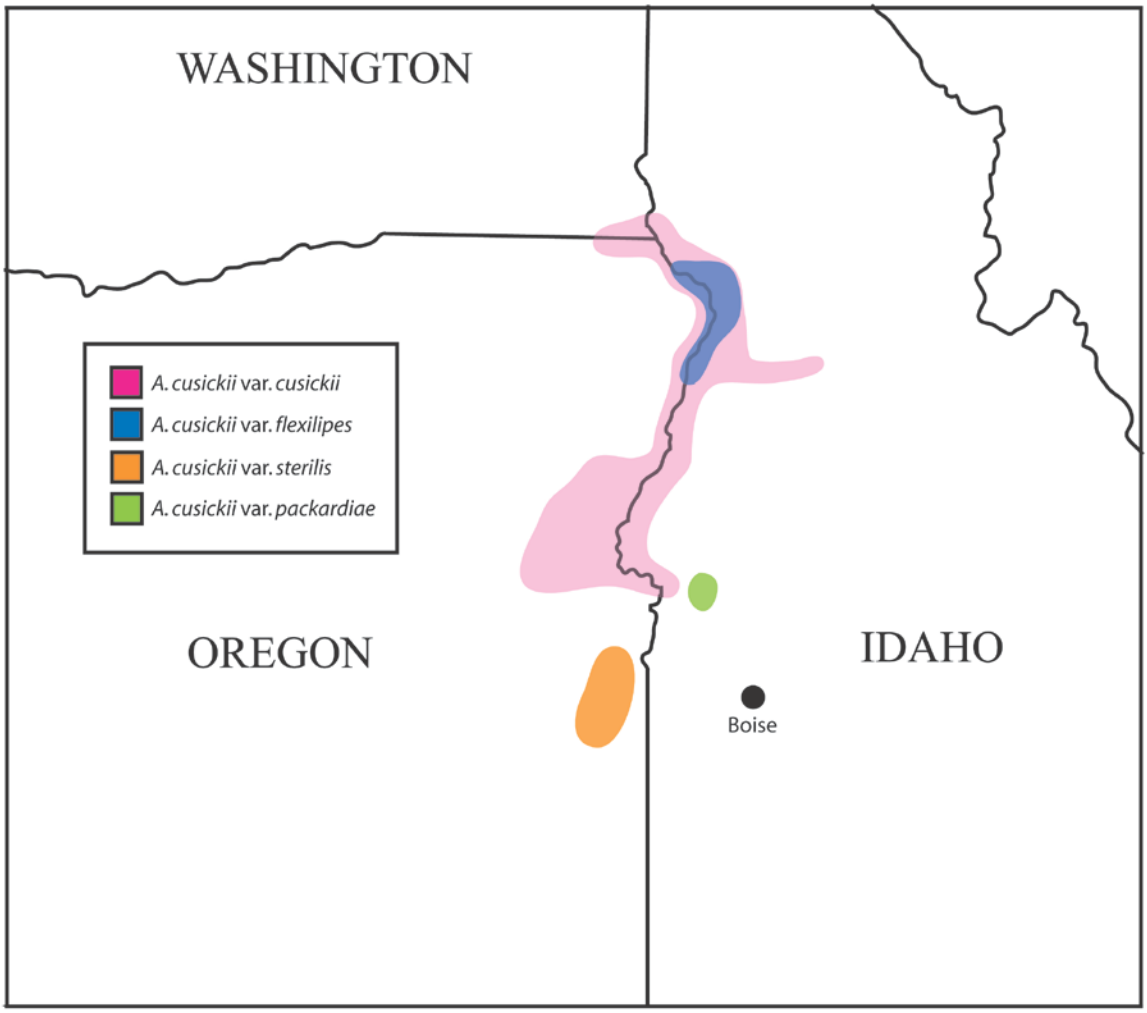


Fig. 2. Map of the approximate ranges of the varieties of *Astragalus cusickii*, focused on an area spanning the borders between the states of Idaho, Oregon, and Washington, in the Pacific northwest region of the United States. Colors corresponding to particular varieties are defined in the inset legend.

Fig. 3. Conspicuous oblique, half-ellipsoid, papery pods on an individual *Astragalus cusickii* var. *flexilipes*, photographed on 27 June 2013 on a steep, sandy slope near the top of a hill in Hells Canyon, Adams county, Idaho.

Fig. 4. Conspicuous inflated, brightly-mottled, papery pods on an individual *Astragalus cusickii* var. *sterilis*, photographed on 11 June 2013 near Birch creek, Malheur county, Oregon.

Fig. 5. Individual *Astragalus cusickii* var. *packardiae* photographed on 30 May 2014 on a hillside in Payette county, Idaho. Numerous slender pods are evident.

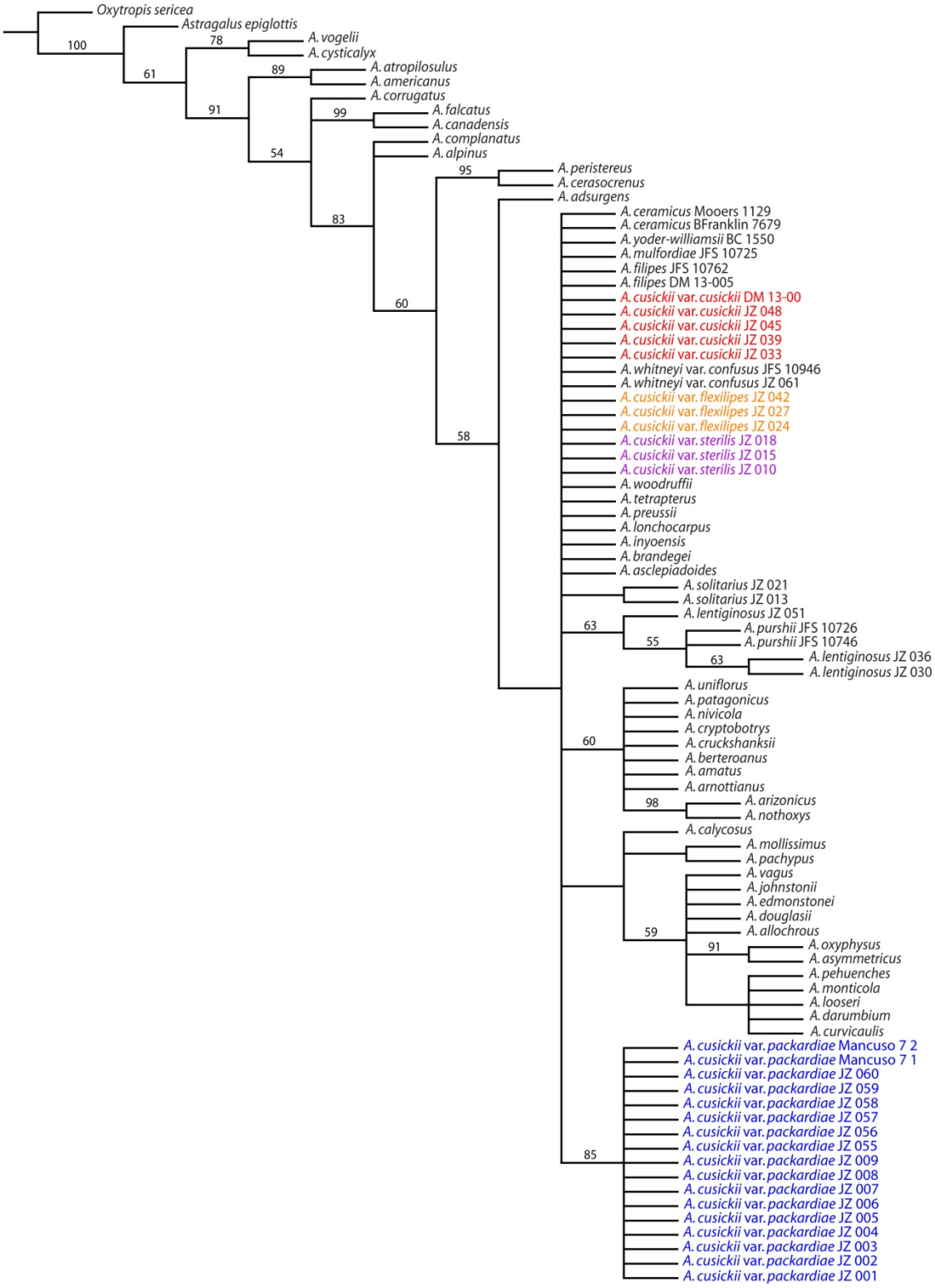


Fig. 6. Strict consensus tree from maximum parsimony analysis of *ITS*, with bootstrap values above branches. Varieties of *Astragalus cusickii* are highlighted in color. 70 equally most-parsimonious trees were found. L = 381, CI = 0.750, RI = 0.806

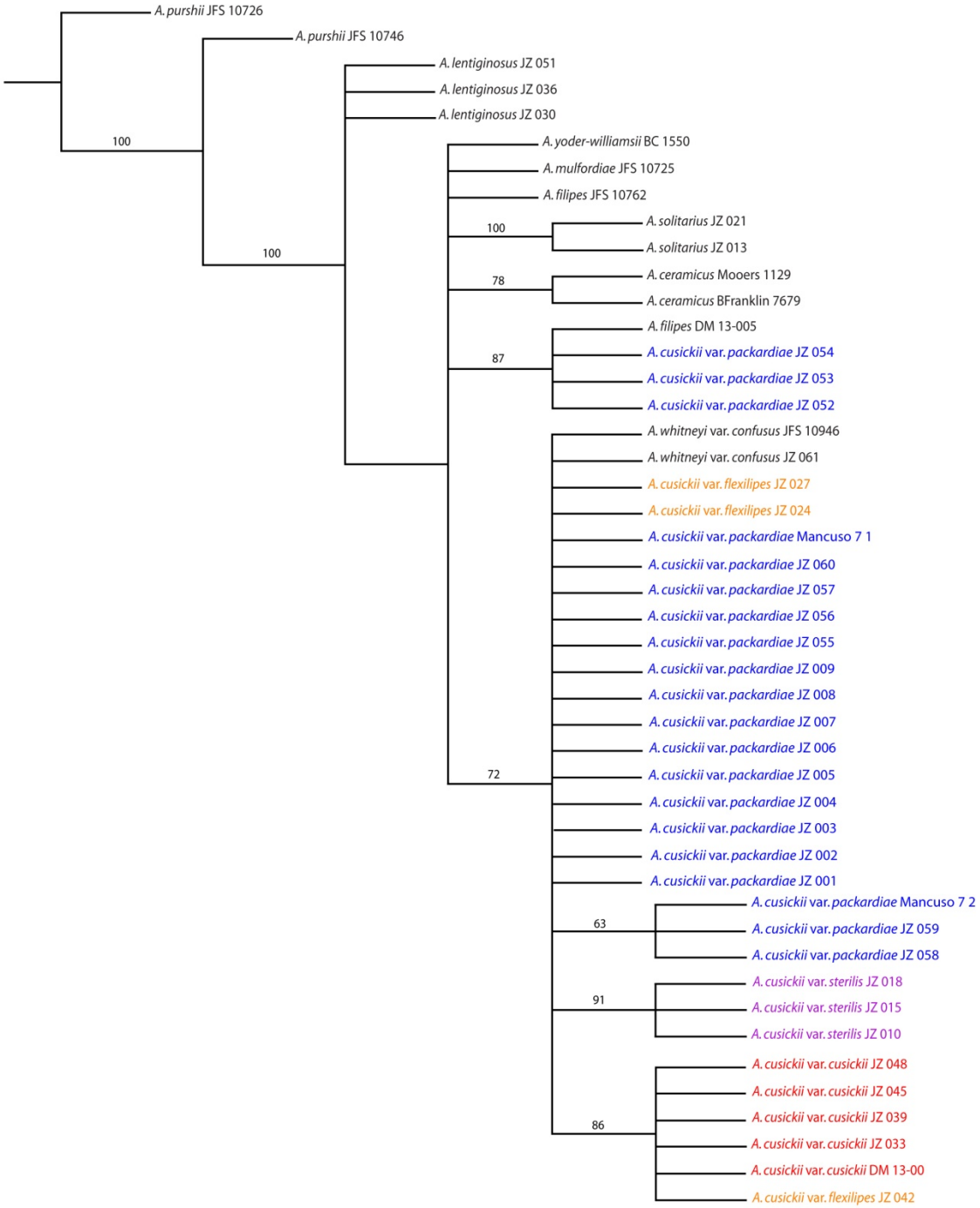


Fig. 7. Strict consensus tree from maximum parsimony analysis of *trnS-G*, with bootstrap values above branches. Varieties of *Astragalus cusickii* are highlighted in color. 3 equally most-parsimonious trees were found. L = 88, CI = 0.977, RI = 0.991

Fig. 8. Strict consensus tree from maximum parsimony analysis of matrix 1, with bootstrap values above branches. Varieties of *Astragalus cusickii* are highlighted in color. 30 equally most-parsimonious trees were found. L = 689, CI = 0.805, RI = 0.869

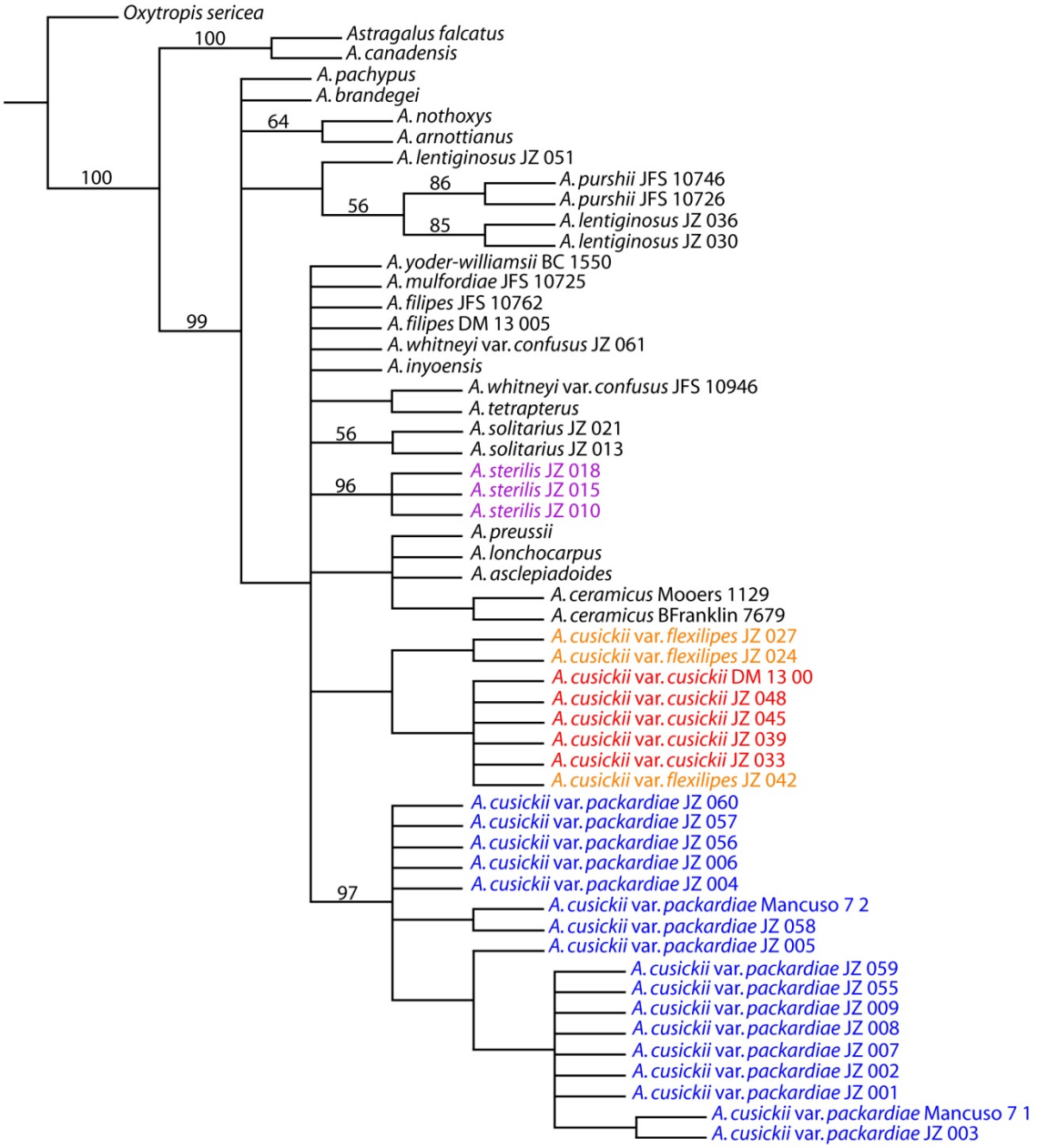


Fig. 9. Strict consensus tree from maximum parsimony analysis of matrix 2, with bootstrap values above branches. Varieties of *Astragalus cusickii* are highlighted in color. 20 equally most-parsimonious trees were found. L = 419, CI = 0.886, RI = 0.929

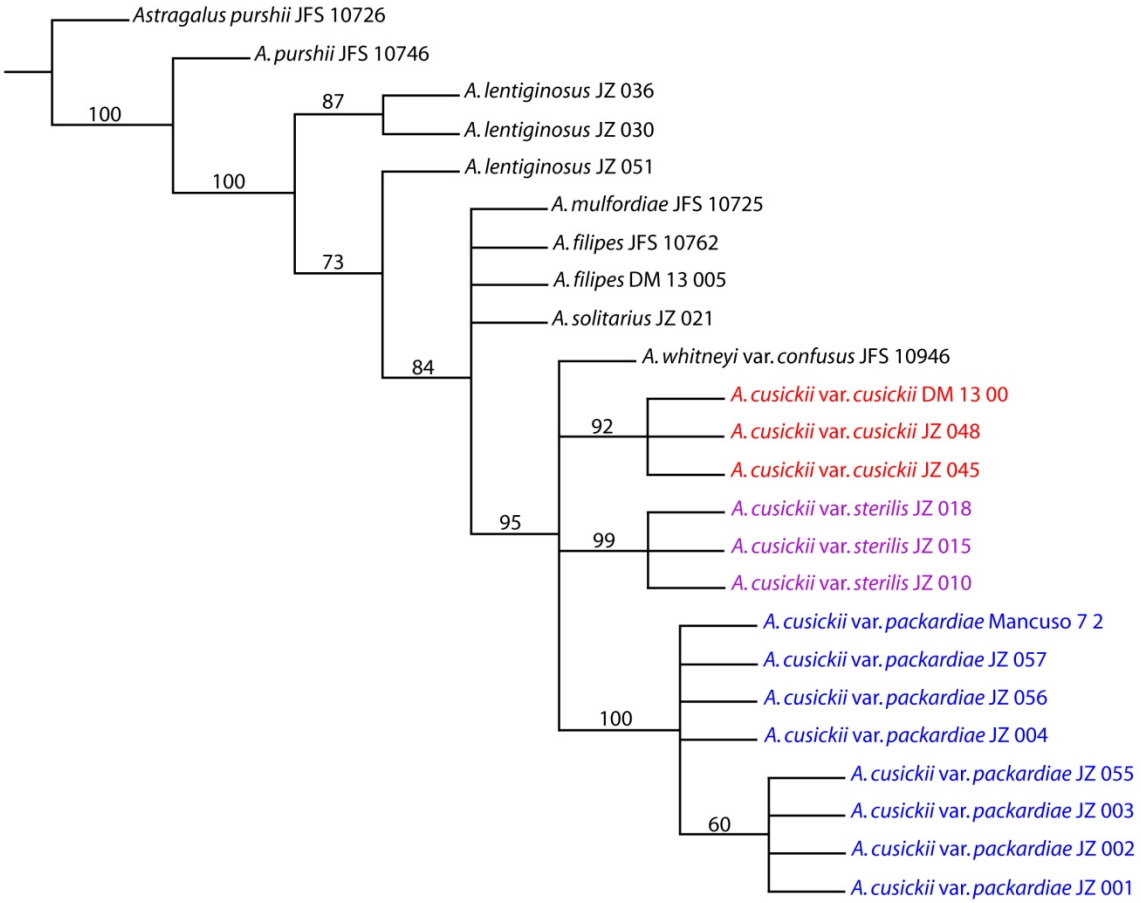


Fig. 10. Strict consensus tree from maximum parsimony analysis of matrix 3, with bootstrap values above branches. Varieties of *Astragalus cusickii* are highlighted in color. A single most-parsimonious tree was found. L = 154, CI = 0.883, RI = 0.941

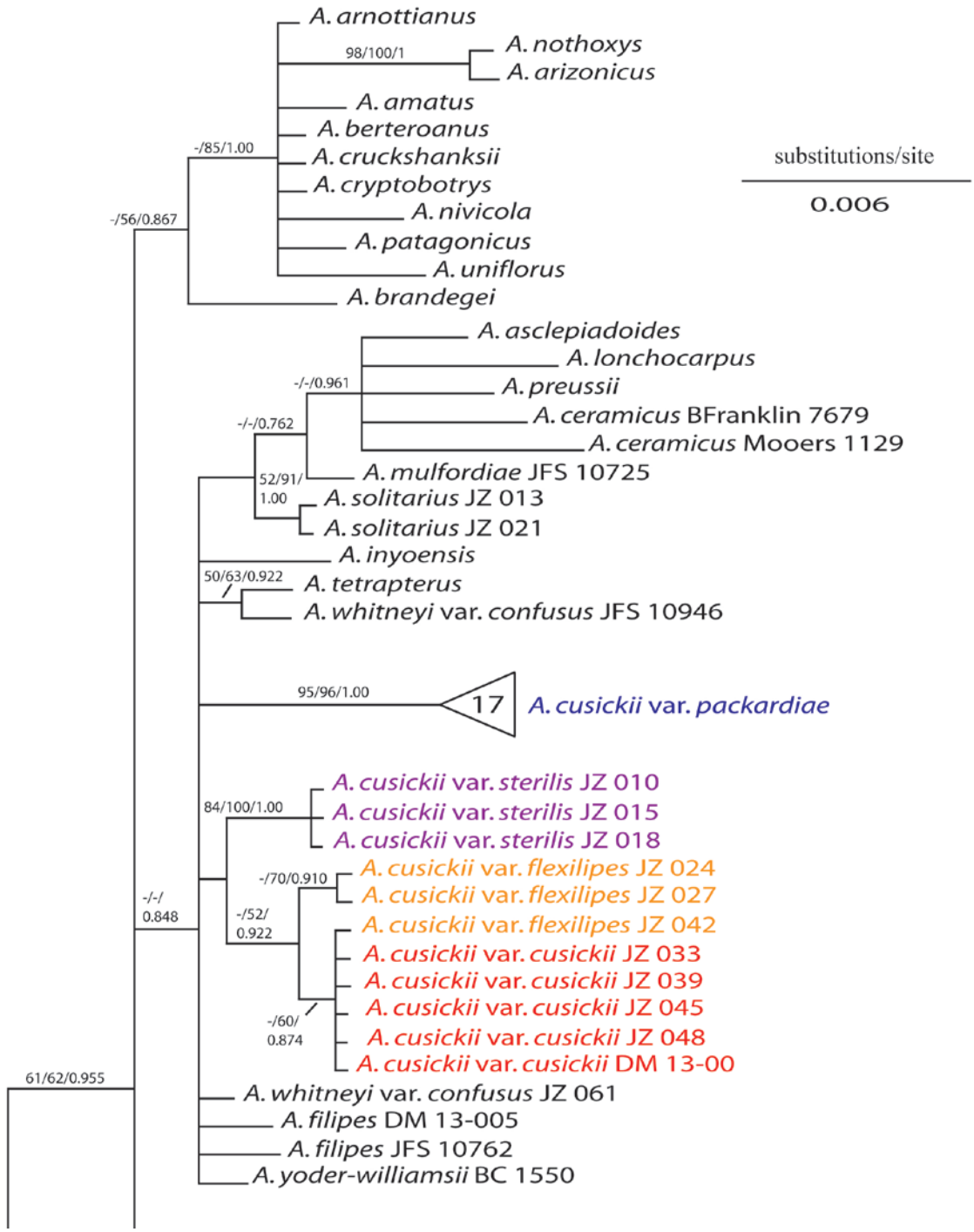
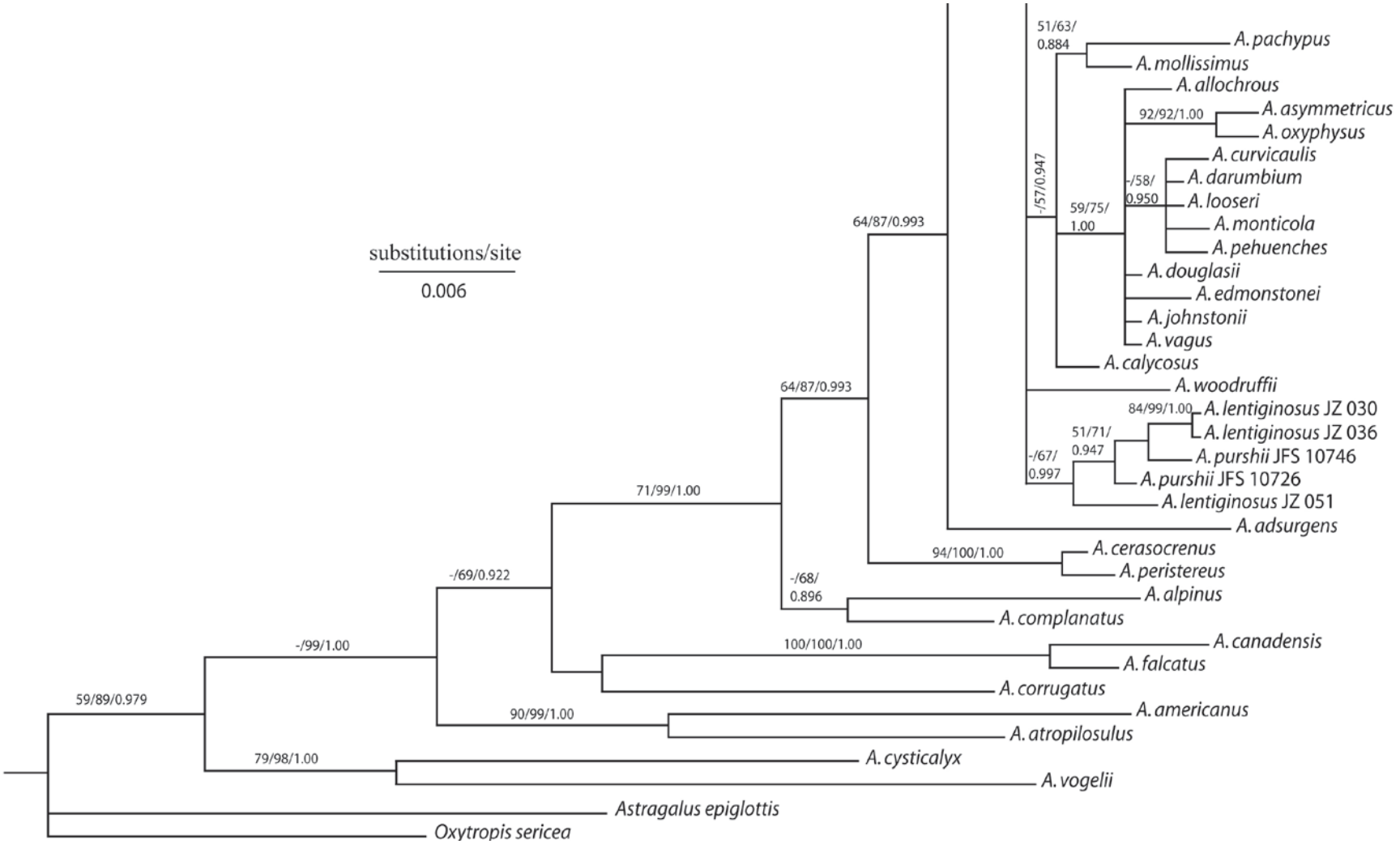


Fig. 11. Majority-rule tree generated from Bayesian inference analysis that is congruent with maximum parsimony and maximum likelihood analyses. Varieties of *Astragalus cusickii* are highlighted in color. Values above branches correspond to maximum parsimony bootstrap support, maximum likelihood bootstrap support, and Bayesian inference posterior probability, respectively. Triangle represents 17 individual *Astragalus cusickii* var. *packardiae* collapsed to save space. Continued on next page.


substitutions/site
0.006
51/63/
0.884
A. pachypus
A. mollissimus
A. allochrous
92/92/1.00
A. asymmetricus
A. oxyphysus
A. curvicaulis
-/58/
0.950
A. darumbium
A. looseri
-/57/0.947
59/75/
1.00
A. monticola
A. pehuenches
64/87/0.993
A. douglasii
A. edmonstonei
A. johnstonii
A. vagus
A. calycosus
64/87/0.993
A. woodruffii
84/99/1.00
A. lentiginosus JZ 030
A. lentiginosus JZ 036
51/71/
0.947
A. purshii JFS 10746
-/67/
0.997
A. purshii JFS 10726
A. lentiginosus JZ 051
71/99/1.00
A. adsurgens
94/100/1.00
A. cerasocrenus
A. peristereus
-/69/0.922
-/68/
0.896
A. alpinus
A. complanatus
-/99/1.00
100/100/1.00
A. canadensis
A. falcatus
A. corrugatus
59/89/0.979
90/99/1.00
A. americanus
A. atropilosulus
79/98/1.00
A. cysticalyx
A. vogelii
Astragalus epiglottis
Oxytropis sericea

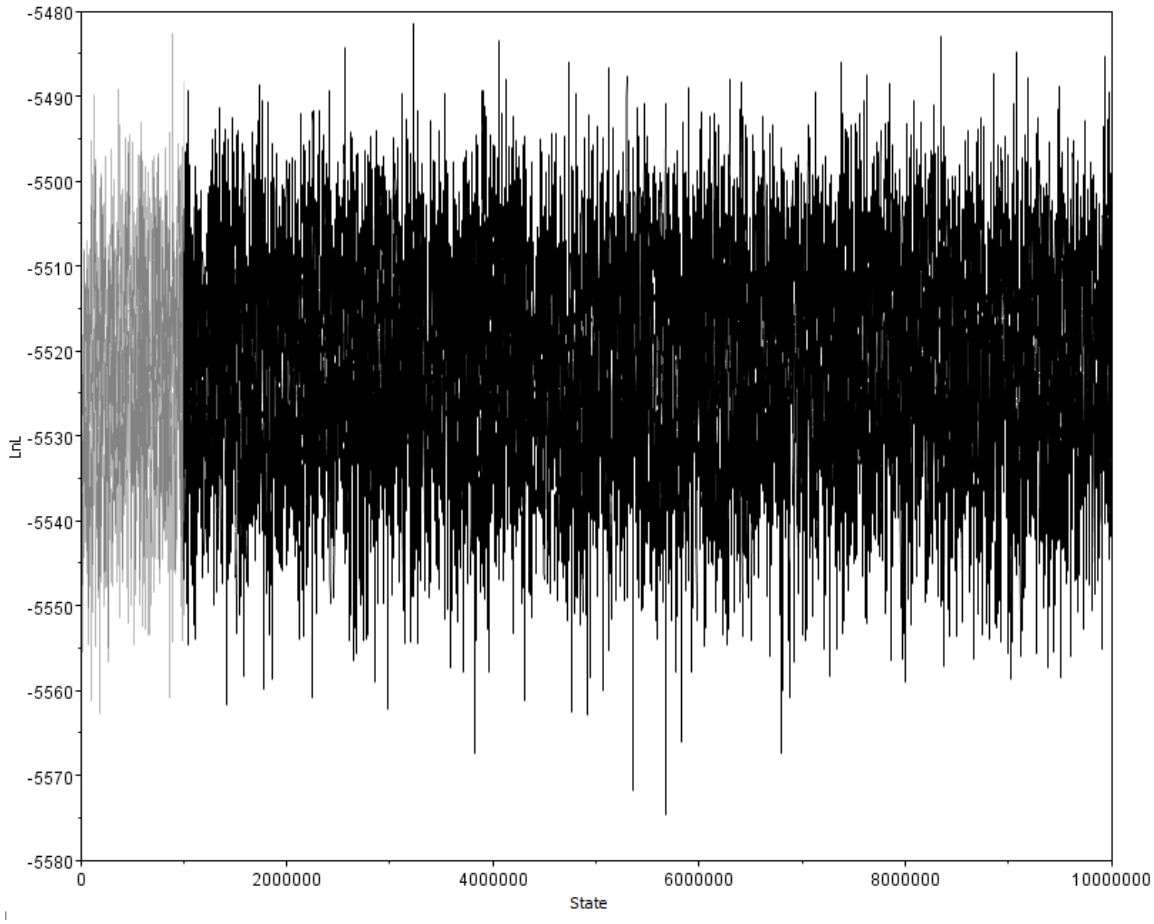


Fig. 12. Combined Metropolis-coupled Markov chain trace plot of independent Bayesian inference analyses of *ITS*, *ETS*, *CNGC4*, and *trnS-G* gene regions in varieties of *Astragalus cusickii* and related species, ran for ten million generations. Burn-in was set at 50,000 generations. X-axis corresponds to generation number. Lack of a clear vertical trend in the data supports MCMC completion.

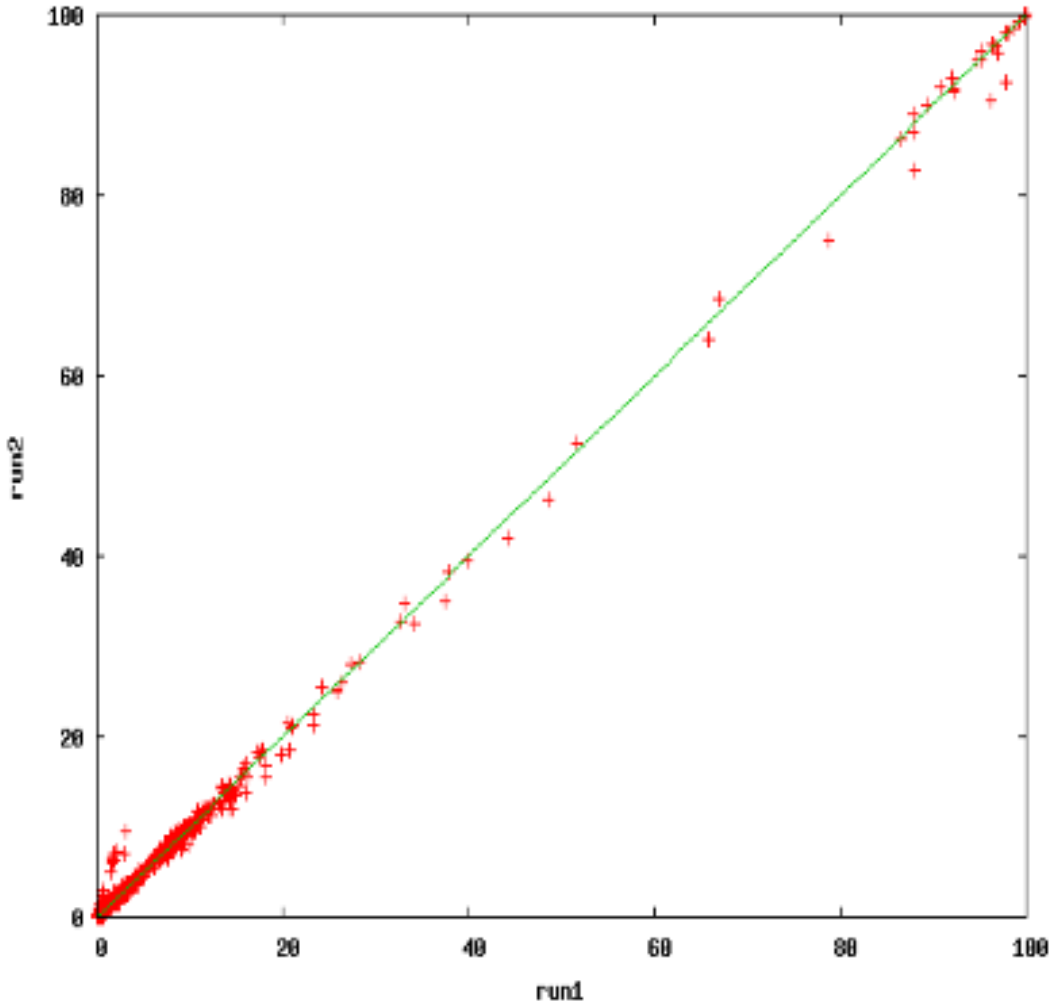


Fig. 13. Joint-marginal plot comparing two independent Bayesian inference analyses of *Astragalus* species using *ITS*, *ETS*, *CNGC4*, and *trnS-G* gene regions. Analyses ran for ten million generations. Metropolis-coupled Markov chain convergence is indicated by the proximity of data points to the diagonal.

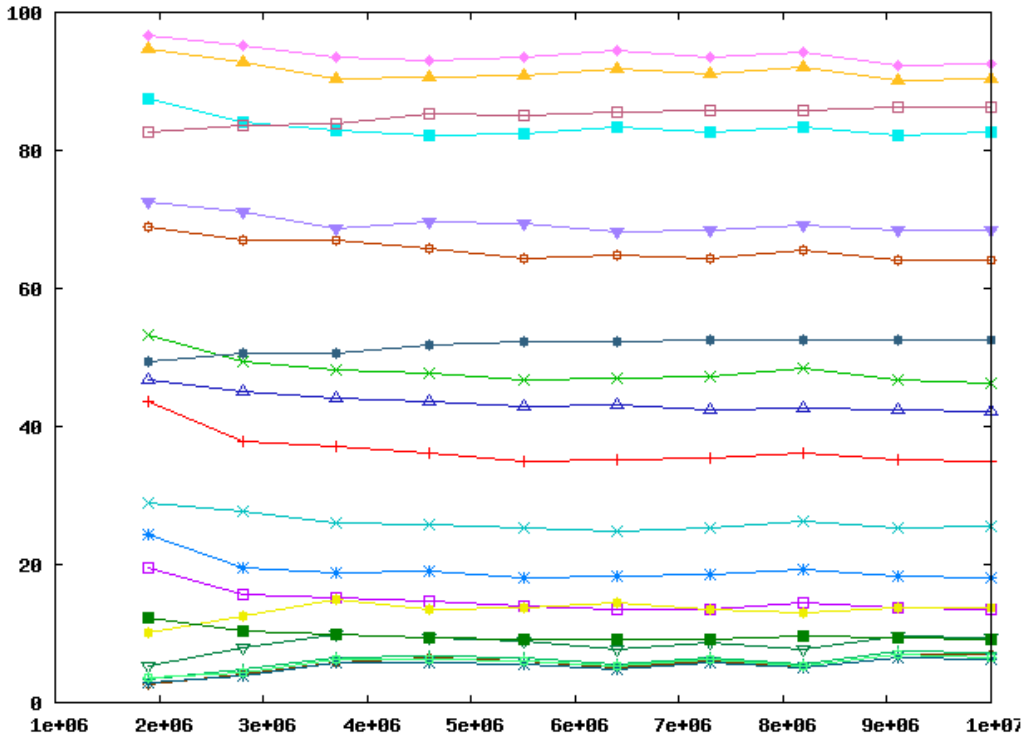


Fig. 14. Are We There Yet plot of Bayesian inference analyses of *ITS*, *ETS*, *CNGC4*, and *trnS-G* gene regions in varieties of *Astragalus cusickii* and related species, ran for ten million generations. Burn-in was set at 50,000 generations. Horizontal tracks indicate MCMC completion.

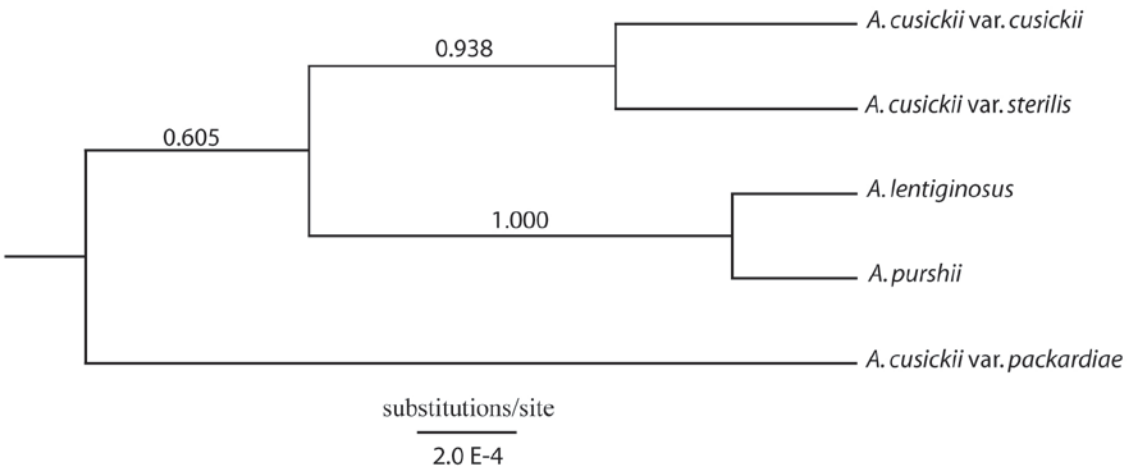


Fig. 15. Species tree resulting from multispecies coalescent analysis conducted in *BEAST including only taxa for which sequence data from more than one individual was available, and where each individual had sequence data for *ITS, ETS, CNGC4,* and *trnS-G* for one billion generations. Posterior probabilities are listed above branches. Note that *BEAST does not require an outgroup to be specified.

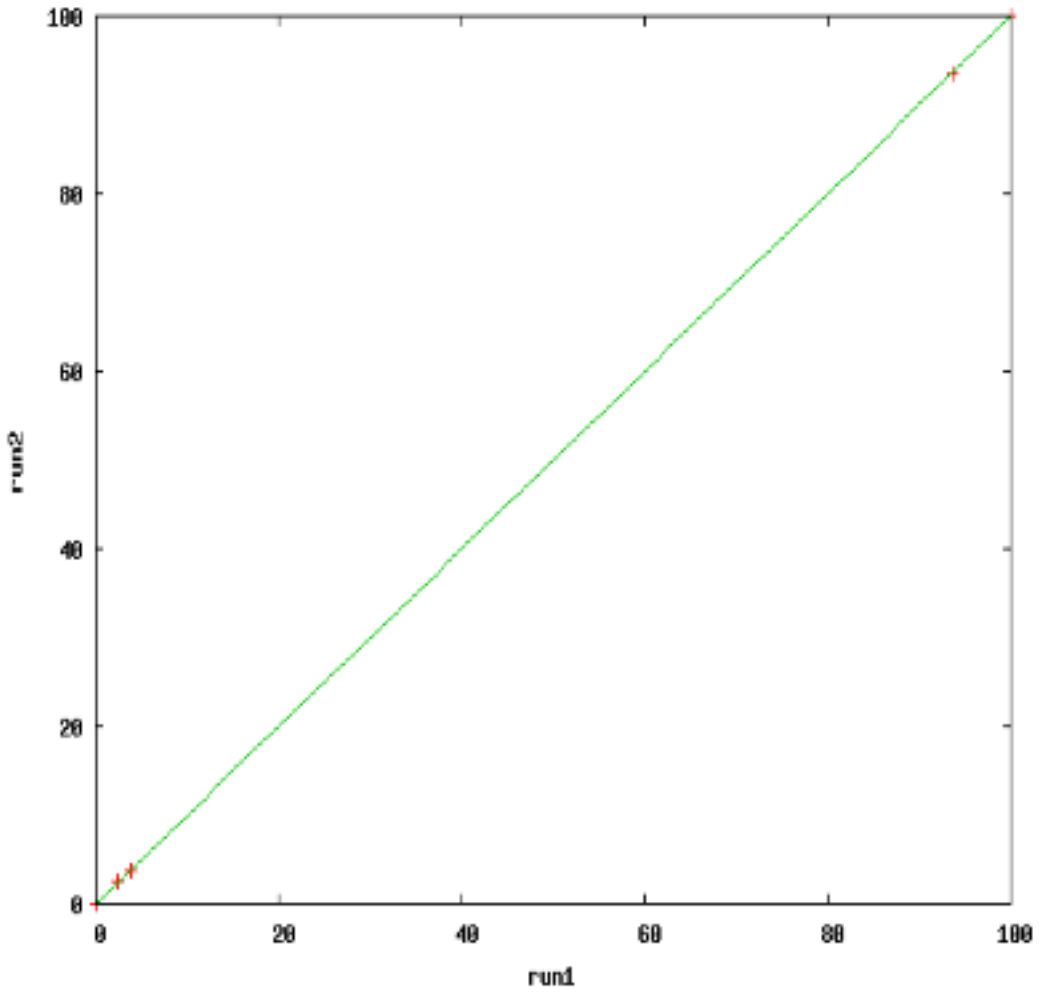


Fig. 16. Joint-marginal plot comparing two independent *BEAST analyses of *Astragalus* species using *ITS, ETS, CNGC4,* and *trnS-G* gene regions. Analysis used the JC69 substitution model and a strict clock for one billion generations. Metropolis-coupled Markov chain convergence is indicated by the proximity of data points to the diagonal.

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APPENDIX

Authority, Voucher, Collection, and GenBank Information Pertaining to Individuals Included in Analyses

Table A.1. Authority, voucher, collection, and GenBank information pertaining to individuals included in analyses.

Individual ID	Voucher ID	Voucher Location	Collected From	Internal Transcribed Spacer GenBank Accession (two labels indicate GenBank sequence split into ITS1 and ITS2)	External Transcribed Spacer GenBank Accession	trnS-trnG GenBank Accession	Cyclic Nucleotide-Gated Channel 4 GenBank Accession	Collection Coordinates
A. cusickii A. Gray var. *packardiae* Barneby JZ 001	*Jay Zimmers 001*	SRP	Payette Co., Idaho	KT202434	KT202406	KT202483	KT202369	44.07728, -116.59731
A. cusickii A. Gray var. *packardiae* Barneby JZ 002	*Jay Zimmers 001*	SRP	Payette Co., Idaho	KT202435	KT202407	KT202484	KT202370	44.07728, -116.59731
A. cusickii A. Gray var. *packardiae* Barneby JZ 003	*Jay Zimmers 001*	SRP	Payette Co., Idaho	KT202436	KT202408	KT202485	KT202371	44.07728, -116.59731
A. cusickii A. Gray var. *packardiae* Barneby JZ 004	*Jay Zimmers 001*	SRP	Payette Co., Idaho	KT202437	KT202409	KT202486	KT202372	44.07728, -116.59731
A. cusickii A. Gray var. *packardiae* Barneby JZ 005	*Jay Zimmers 001*	SRP	Payette Co., Idaho	KT202438		KT202487	KT202373	44.07728, -116.59731
A. cusickii A. Gray var. *packardiae* Barneby JZ 006	*Jay Zimmers 006*	SRP	Payette Co., Idaho	KT202439		KT202488	KT202374	44.07257, -116.59618
A. cusickii A. Gray var. *packardiae* Barneby JZ 007	*Jay Zimmers 006*	SRP	Payette Co., Idaho	KT202440		KT202489	KT202375	44.07257, -116.59618
A. cusickii A. Gray var. *packardiae* Barneby JZ 008	*Jay Zimmers 006*	SRP	Payette Co., Idaho	KT202441		KT202490	KT202376	44.07257, -116.59618
A. cusickii A. Gray var. *packardiae* Barneby JZ 009	*Jay Zimmers 006*	SRP	Payette Co., Idaho	KT202442		KT202491	KT202377	44.07257, -116.59618
A. cusickii A. Gray var. *packardiae* Barneby JZ 052	*Jay Zimmers 052*	SRP	Payette Co., Idaho	KT202443				44.06817, -116.64630
A. cusickii A. Gray var. *packardiae* Barneby JZ 053	*Jay Zimmers 052*	SRP	Payette Co., Idaho	KT202444			KT202378	44.06817, -116.64630
A. cusickii A. Gray var. *packardiae* Barneby JZ 054	*Jay Zimmers 052*	SRP	Payette Co., Idaho	KT202445			KT202379	44.06817, -116.64630
A. cusickii A. Gray var. *packardiae* Barneby JZ 055	*Jay Zimmers 055*	SRP	Payette Co., Idaho	KT202446	KT202410	KT202492	KT202380	44.08755, -116.59938
A. cusickii A. Gray var. *packardiae* Barneby JZ 056	*Jay Zimmers 055*	SRP	Payette Co., Idaho	KT202447	KT202411	KT202493	KT202381	44.08755, -116.59938
A. cusickii A. Gray var. *packardiae* Barneby JZ 057	*Jay Zimmers 055*	SRP	Payette Co., Idaho	KT202448	KT202412	KT202494	KT202382	44.08755, -116.59938
A. cusickii A. Gray var. *packardiae* Barneby JZ 058	*Jay Zimmers 058*	SRP	Payette Co., Idaho	KT202449		KT202495	KT202383	44.08137, -116.57037
A. cusickii A. Gray var. *packardiae* Barneby JZ 059	*Jay Zimmers 058*	SRP	Payette Co., Idaho	KT202450		KT202496	KT202384	44.08137, -116.57037
A. cusickii A. Gray var. *packardiae* Barneby JZ 060	*Jay Zimmers 058*	SRP	Payette Co., Idaho	KT202451		KT202497	KT202385	44.08137, -116.57037
A. cusickii A. Gray var. *packardiae* Barneby Mancuso 7-1	not vouchered	N/A	Payette Co., Idaho	KT202461		KT202498	KT202386	
A. cusickii A. Gray var. *packardiae* Barneby Mancuso 7-2	not vouchered	N/A	Payette Co., Idaho	KT202462	KT202420	KT202499	KT202387	
A. cusickii A. Gray var. *sterilis* (Barneby) Barneby JZ 010	*Jay Zimmers 010*	SRP	Malheur Co., Oregon	KT202452	KT202413	KT202500	KT202388	43.20928, -117.50368
A. cusickii A. Gray var. *sterilis* (Barneby) Barneby JZ 015	*Jay Zimmers 015*	SRP	Malheur Co., Oregon	KT202453	KT202414	KT202501	KT202389	43.23320, -117.49730
A. cusickii A. Gray var. *sterilis* (Barneby) Barneby JZ 018	*Jay Zimmers 018*	SRP	Malheur Co., Oregon	KT202454	KT202415	KT202502	KT202390	43.29102, -117.10195
A. solitarius M.E. Peck JZ 013	*Jay Zimmers 013*	SRP	Malheur Co., Oregon	KT202458		KT202511	KT202399	42.79220, -117.59850
A. solitarius M.E. Peck JZ 021	*Jay Zimmers 021*	SRP	Malheur Co., Oregon	KT202459	KT202416	KT202512	KT202400	42.85647, -117.71932
A. cusickii A. Gray var. *flexilipes* Barneby JZ 024	*Jay Zimmers 024*	SRP	Adams Co., Idaho	KT202431		KT202480	KT202366	45.14390, -116.70790
A. cusickii A. Gray var. *flexilipes* Barneby JZ 027	*Jay Zimmers 027*	SRP	Adams Co., Idaho	KT202432		KT202481	KT202367	45.14570, -116.71450

A. cusickii A. Gray var. *flexilipes* Barneby JZ 042	*Jay Zimmers 042*	SRP	Washington Co., Idaho	KT202433		KT202482	KT202368	44.60650, -117.06919
A. lentiginosus Dougl. ex Hook. JZ 030	*Jay Zimmers 030*	SRP	Malheur Co., Oregon	KT202455	KT202417	KT202505	KT202393	44.25950, -117.58140
A. lentiginosus Dougl. ex Hook. JZ 036	*Jay Zimmers 036*	SRP	Malheur Co., Oregon	KT202456	KT202418	KT202506	KT202394	44.24834, -117.59965
A. lentiginosus Dougl. ex Hook. JZ 051	*Jay Zimmers 051*	SRP	Washington Co., Idaho	KT202457	KT202419	KT202507	KT202395	44.59935, -117.09200
A. whitneyi A. Gray var. *confusus* Barneby JZ 061	*Jay Zimmers 061*	SRP	Harney Co., Oregon	KT202460		KT202514	KT202402	42.66608, -118.56521
A. whitneyi A. Gray var. *confusus* Barneby JFS 10946	*James F Smith 10946*	SRP		KT202463	KT202421	KT202513	KT202401	
A. cusickii A. Gray var. *cusickii* JZ 033	*Jay Zimmers 033*	SRP	Malheur Co., Oregon	KT202427		KT202476	KT202362	44.25042, -117.60001
A. cusickii A. Gray var. *cusickii* JZ 039	*Jay Zimmers 039*	SRP	Washington Co., Idaho	KT202428		KT202477	KT202363	44.52079, -117.17361
A. cusickii A. Gray var. *cusickii* JZ 045	*Jay Zimmers 045*	SRP	Washington Co., Idaho	KT202429	KT202405	KT202478	KT202364	44.59935, -117.09200
A. cusickii A. Gray var. *cusickii* JZ 048	*Jay Zimmers 048*	SRP	Washington Co., Idaho	KT202430	KT202404	KT202479	KT202365	44.56147, -117.13944
A. cusickii A. Gray var. *cusickii* DM 13-100	*Don Mansfield 13-100*	CIC		KT202464	KT202422	KT202475	KT202361	
A. filipes Torr. ex A. Gray DM 13-005	*Don Mansfield 13-005*	CIC		KT202465		KT202503	KT202391	
A. filipes Torr. ex A. Gray JFS 10762	*James F Smith 10762*	SRP		KT202466	KT202423	KT202504	KT202392	
A. mulfordiae M.E. Jones JFS 10725	*James F Smith 10725*	SRP		KT202467	KT202424	KT202508	KT202396	
A. purshii Dougl. ex Hook. JFS 10726	*James F Smith 10726*	SRP		KT202468	KT202425	KT202509	KT202397	
A. purshii Dougl. ex Hook. JFS 10746	*James F Smith 10746*	SRP		KT202469	KT202426	KT202510	KT202398	
A. yoder-williamsii Barneby BC 1550	*Beth Corbin 1550*	SRP		KT202470		KT202515	KT202403	
A. ceramicus E. Sheld. Bfranklin 7679	*B. Franklin 7679*	SRP	Iron Co., Utah	KT202471		KT202473	KT202359	
A. ceramicus E. Sheld. Mooers 1129	*Blaine H.M. Mooers 1129*	SRP	Sheridan Co., Montana	KT202472		KT202474	KT202360	
Oxytropis sericea Nutt.	*Wojciechowski and Sanderson 255*			AF121757			DQ107239	
A. arnottianus Gillies	*Scherson 100*			EU282975			DQ107227	
A. asclepiadoides M.E. Jones	*Sanderson 996*			AF121725			DQ107235	
A. brandegeei Porter	*Wojciechowski and Sanderson 157*			L10768, L10769			DQ107237	
A. canadensis L.	*Wojciechowski and Sanderson 302*			L10770, L10771			DQ107240	
A. falcatus Lam.	*Weber 15359*			U50488, U50489			DQ107241	
A. inyoensis E. Sheld.	*Wojciechowski 527*			AF121737			DQ107232	
A. lonchocarpus Torr.	*Wojciechowski and Sanderson 143*			AF121689			DQ107230	
A. nothoxys A. Gray	*Wojciechowski and Sanderson 177*			AF121688			DQ107231	
A. pachypus Greene	*Sanderson 984*			AF121722			DQ107236	
A. preussii A. Gray	*Sanderson 999*			AF121726			DQ107234	
A. tetrapterus A. Gray	*Sanderson 1006*			AF121728			DQ107228	
A. adsurgens Pall.	*Wojciechowski and Sanderson 267*			AF121674				
A. allochrous A. Gray	*Sanderson 953*			AF121707				
A. alpinus L.	not vouchered			HQ613380				
A. amatus Clos.	*Scherson 106*			EU282976				
A. americanus M.E. Jones	not vouchered			U50492, U50493				

A. arizonicus A. Gray	*Sanderson 968*			AF121690				
A. asymmetricus E. Sheld.	*Sanderson 981*			AF121710				
A. atropilosulus Hochst.	*Yamashita et al. 1068*			AB051939				
A. berteroanus Moris	*Scherson 113*			EU282983				
A. calycosus Torr. ex S. Watson	*Sanderson 975*			AF121691				
A. cerasocrenus Bunge	not vouchered			U50514				
A. complanatus R. Br. ex Bunge	not vouchered			EU591995				
A. corrugatus Bertol.	not vouchered			HQ613378				
A. cruckshanksii Hook & Arn.	*Scherson 101*			EU282989				
A. cryptobotrys I.M. Johnst.	*Scherson 108*			EU282980				
A. curvicaulis Clos.	*Scherson 112*			EU282984				
A. cysticalyx Ledeb.	*Liston 961*			AF121682				
A. darumbium Bertero ex Colla	*Scherson 105*			EU282973				
A. douglasii Torr. & A. Gray	*Sanderson 980*			AF121709				
A. edmonstonei (Hook. f.) B.L. Rob.	*Scherson 110*			EU282978				
A. epiglottis L.	*Podlech 45851*			AB051910				
A. johnstonii Gomez-Sosa	*Scherson 102*			EU282988				
A. looseri I.M. Johnst.	*Scherson 104*			EU282974				
A. mollissimus Torr.	*Sanderson 950*			AF121719				
A. monticola Phil.	*Scherson 103*			EU282982				
A. nivicola Gomez-Sosa	*Scherson 111*			EU282985				
A. oxyphysus A. Gray	*Sanderson 979*			AF121708				
A. patagonicus Phil.	*Sanderson 2515*			AF121746				
A. pehuenches Niederl.	*Scherson 107*			EU282981				
A. peristereus Boiss. & Hausskn.	not vouchered			U50494, U50495				
A. uniflorus DC.	not vouchered			EU282986				
A. vagus Reiche	*Scherson 109*			EU282979				
A. vogelii (Webb) Bornm.	*Mozaffarian et.al. 39103*			AB051911				
A. woodruffii M.E. Jones	*Sanderson 995*			AF121724				