DNA barcodes reveal microevolutionary signals in fire response trait in two legume genera

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Abstract. Large-scale DNA barcoding provides a new technique for species identification and evaluation of relationships across various levels (populations and species) and may reveal fundamental processes in recently diverged species. Here, we analysed DNA sequence variation in the recently diverged legumes from the Psoraleeae (Fabaceae) occurring in the Cape Floristic Region (CFR) of southern Africa to test the utility of DNA barcodes in species identification and discrimination. We further explored the phylogenetic signal on fire response trait (reseeding and resprouting) at species and generic levels. We showed that Psoraleoid legumes of the CFR exhibit a barcoding gap yielding the combination of matK and rbcLa (matK + rbcLa) data set as a better barcode than single regions. We found a high score (100 %) of correct identification of individuals to their respective genera but a very low score (< 50 %) in identifying them to species. We found a considerable match (54 %) between genetic species and morphologically delimited species. We also found that different lineages showed a weak but significant phylogenetic conservatism in their response to fire as reseeders or resprouters, with more clustering of resprouters than would be expected by chance. These novel microevolutionary patterns might be acting continuously over time to produce multi-scale regularities of biodiversity. This study provides the first insight into the DNA barcoding campaign of land plants in species identification and detection of the phylogenetic signal in recently diverged lineages of the CFR.

Keywords: Fabaceae; Otholobium; Psoralea; reseeders; resprouters; South Africa.

Introduction

The primary goal of DNA barcoding is the identification of an unknown sample by correctly matching a specific genetic marker to a reference sequence library. However, DNA barcoding can also be used as a tool for addressing fundamental questions in ecology, evolution and conservation biology (Kress et al. 2015). For evolutionary biologists and ecologists, one of the goals of DNA barcoding is to understand the origin of species and the factors causing the difference in species richness in different biomes across the globe. Generally, the full diversity of species in most diverse habitats is still poorly known (Kress et al. 2015). The primary focus of this article is to explore the application of DNA barcoding in some recently diverged lineages of an
exceptionally unique fire derived biodiversity hotspot to determine its efficacy in species identification and detection of microevolutionary signals.

The Greater Cape Floristic Region (GCFR) is a home to Fynbos and the Succulent Karoo biomes—two major biodiversity hotspots located in the winter rainfall area of southern Africa (Myers et al. 2000) (Fig. 1). The Fynbos biome (also called the CFR) is famed for its high species diversity consisting of ~9000 species of vascular plants packed into an area of 90,760 km² of which ~69 % are endemic (Manning and Goldblatt 2012). The family Fabaceae consists of ~764 species in 43 genera. It is the second largest family in the CFR flora after Asteraceae. Three of the major clades of Fabaceae include the Crotalarieae (300 species), Podalyrieae (125 species) and African Psoraleeae (120 species). These legume lineages differ in their patterns of diversification, with Crotalarieae and Podalyrieae originating in the Eocene ca. 40 Ma (Edwards and Hawkins 2007; Schnitzler et al. 2011) and the African Psoraleeae originating during the Pliocene ca. 5 Ma (Egan and Crandall 2008). This suggests that the African Psoraleeae is a young lineage, which has undergone rapid recent radiation giving rise to ~75 species of Psoralea L. and ~53 species of Otholobium C.H.Stirt. (Stirton 2005; Manning and Goldblatt 2012). Majority of species in Otholobium and Psoralea have a narrow distribution and are frequently restricted to a single mountain stream or slope or a single soil type. In addition, several species are listed in the IUCN Red List under different levels of conservation categories ranging from extinct in the wild (e.g. Psoralea gueinzii and P. cataracta) to endangered (e.g. Otholobium bowieanum, O. incanum, P. fascicularis and P. filifolia) and vulnerable (O. hamatum, O. venustum, P. abbottii and P. alata) (Raimondo et al. 2009).

Fynbos is a fire prone vegetation that requires regular burning for its persistence. The high species richness in the Fynbos biome has been ascribed to fire (Cowling et al. 1996; Linder 2003; Power et al. 2011). Plants adapt to fires in two major ways: as resprouters or reseeders (Bell 2001). Resprouting plants survive fire as individuals and then replace the lost structures by resprouting from surviving tissues. Conversely, reseeding individuals are often killed by fire (Fig. 2) and the population is re-established by a new generation growing from seeds (Bell 2001). Fire-survival and regeneration strategies of plants have been the subject of numerous studies (e.g. Keeley 1977; Bond 1985; Le Maitre and Midgley 1992; Schutte et al. 1995; Pausas and Keeley 2014; Scott et al. 2014). Cowling (1987) postulated that the high species diversity in the Gondwanan floras (Australian kwongan and Cape fynbos) may be ascribed to recurrent fires, edaphic specialization and short dispersal distance. There are noticeable differences in the allocation of resources to reserve storage, vegetative growth and

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Figure 1. Map of the GCFR showing the Fynbos and the Succulent Karoo Biomes constructed based on Mucina and Rutherford (2006).
reproductive effort linked with these fire-survival strategies (Bond and van Wilgen 1996; Bell 2001; Bond and Midgley 2001; Scott et al. 2014). For example, while reseeders are generally characterized by a shorter lifespan, they tend to grow rapidly and taller with much allocation of resources predominantly above ground. Resprouters, on the other hand, have longer lifespans, slower growth, produce fewer seeds and have a below ground resource allocation in starch-rich lignotubers (Hansen et al. 1991; Bell and Ojeda 1999). Reseedsers produce larger numbers of viable seeds than do resprouters due to their greater reliance on seed for survival (Hansen et al. 1991; Bell 2001), resulting in elevated post-fire recruitment. There are also reported differences in seed yield and quality with reseedsers having higher N and P concentrations in the seeds than those of congenetic resprouters (Hansen et al. 1991). Other differences include nutritional requirements with reseedsers requiring more nutrients than the resprouters due to the high nutritional costs of seed production and growth (Hansen et al. 1991; Bell 2001). These strategies influence speciation rates in woody genera in the fynbos (Wells 1969; Litsios et al. 2014), with reseedsers shown to have higher diversification rates than resprouters (Litsios et al. 2014). Other studies have shown that fire-survival and regeneration strategy (reseeding/resprouting) is a character of taxonomic, ecological and evolutionary importance in Fynbos legumes (Schutte et al. 1995; Litsios et al. 2014; Scott et al. 2014).

Traditionally, species identification depends primarily on morphological features (morphospecies). As molecular data became increasingly available and new techniques such as DNA barcoding emerged, species identification is becoming fast, reliable and more accurate. Here, we use matK and rbcLa and the combination of the two regions (matK + rbcLa), based on their recognition as core plant barcode markers by the Consortium for the Barcode of Life Plant Working Group (CBOL 2009) to (i) test their efficacy in identifying species of two southern African Psoraleoid genera (Otholobium and Psoralea); (ii) explore the potential of the DNA barcode markers in grouping Psoraleoid legume sequences into molecular operational taxonomic units (MOTUs) or genetic species units and (iii) test the power of DNA barcodes in revealing microevolutionary patterns including fire-survival and regeneration strategies. The genera Otholobium and Psoralea were chosen for this study because they both have species with reseeding and resprouting modes of regeneration (Fig. 3). Furthermore, although the two genera are closely related (Dludlu et al. 2013), they differ markedly in terms of their morphology and ecology. For example, Otholobium species differ from Psoralea by the absence of a cupulum on the flower pedicel (unique structure in Psoralea, Tucker and Stirton 1991); possession of entire recurved mucronate-obovate to oblanceolate leaflets and inflorescences characterized by bracteate triplets of flowers, with each triplet subtended by a single variously shaped bract (Stirton 1981). Leaves of Psoralea range from 1- to 19-foliolate compound structures or reduced to small-scale-like structures with only P. aculeata having a recurved mucro (Stirton 1989; Manning and Goldblatt 2012), and each flower is

Figure 2. A recent fire burn in the Cape Fynbos, Table Mountain on 5 March 2015. Photograph: A.B.
subtended by a pair of free minute bracts. The two genera also differ in terms of habitat preferences. Eighty per cent of *Psoralea* species inhabit seeps, marshes, riverbanks and/or moist, mist laden high-altitude habitats, while *Otholobium* species occur predominantly in drier habitats, with only 11 % of species occupying the moist habitats favoured by *Psoralea* (Stirton 1989; Manning and Goldblatt 2012).

**Methods**

**Taxon sampling**

We collected 172 samples representing 26 species of *Otholobium* and 43 species of *Psoralea* across their distribution range in the CFR. Where possible, each species was represented by two or more different samples. In all, we collected 72 samples of *Otholobium* and 100 samples of *Psoralea* (voucher specimens are deposited at the Bolus Herbarium (BOL) and listed in Table 1). Of these samples, 23 out of the 26 species of *Otholobium* and 26 out of 43 species of *Psoralea* are represented by more than one sample. Only samples for which sequences for both genes (*matK* and *rbcLa*) are available were included in the analyses. The final data set used in the analyses included 4 reseeding (27 samples) and 22 resprouting (35 samples) species of *Otholobium*, and 35 (43 samples) reseeding and 8 (56 samples) resprouting species of *Psoralea*. Information on fire response strategy was extracted from Stirton (1989), Manning and Goldblatt (2012) and Snijman (2013). To our knowledge, no species included in our analysis show both fire response strategies in wild populations. Collection details including GPS coordinates, altitude and photographs of specimens are available online in the Barcode of Life Data Systems (BOLD; www.boldsystems.org) together with DNA sequences.

**DNA extraction, sequencing and alignment**

All the samples were sent to the Canadian Centre for DNA Barcoding (CCDB) in Canada, where total DNA was extracted and the two core DNA barcodes (*matK* and *rbcLa*) were sequenced according to standard CCDB protocols (Ivanova et al. 2006). Sequence alignment was performed using Multiple Sequence Comparison by Log Expectation (MUSCLE v. 3.8.31, Edgar 2004) plugin in Geneious v.8.0.4 (Kearse et al. 2012) and manually adjusted using MESQUITE v.2.5 (Maddison and Maddison 2008). The two regions were aligned separately and then combined.

**Evaluation of DNA barcodes**

First, we evaluated the performance of the DNA markers (*matK*, *rbcLa* and *matK* + *rbcLa*) in species identification and delimitation of African Psoraleoid legumes at species and generic levels by applying two criteria commonly used to evaluate the utility of the DNA barcodes in species discrimination: the barcode gap of Meyer and Paulay (2005) and discriminatory power (Hebert et al. 2004b). Barcode gap was assessed by comparing intraspecific variation (i.e. the amount of genetic variation within species) to interspecific variation (between species). A good barcode gap was defined as the maximum distance observed within species being at least 2.5 times larger than the mean pairwise distance observed between species.

**Figure 3.** Habitat in *Otholobium* and *Psoralea* species: (A) reseeding, *O. spicatum*; (B) resprouting, *O. rotundifolium*; (C) reseeding, *P. pinnata*; (D) resprouting, *Psoralea* sp. nov. Photographs: C.H.S. (A–C) and A.B. (D).
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should exhibit a significant gap, meaning that sequence variation within species should be significantly lower than between species. Statistical significance between intra- and interspecific variation was assessed using Wilcoxon test in R (R Core Team 2013).

The discriminatory power of DNA barcoding was tested by evaluating the proportion of correct species identification at different taxonomic levels (species and generic) using matK, rbcLa and matK + rbcLa regions. All sequences were labelled according to the names of the species from which the sequences were generated. The test of discriminatory power was carried out using two methods: the ‘best close match’ (Meier et al. 2006) and the ‘near neighbour’ using the functions bestCloseMatch and nearNeighbour implemented in the R package Spider (Brown et al. 2012). Before the test, we determined the optimized genetic distance suitable as threshold for taxon identification using the function localMinima also implemented in Spider (Brown et al. 2012).

The function bestCloseMatch conducts the ‘best close match’ analysis (Meier et al. 2006) by searching for the closest individual in the data set. If the closest individual is within a given threshold, the outcome is scored as ‘correct’, and if it is further, then the result is ‘no ID’ (no identification). If more than one species is tied for closest match, the outcome of the test is an ‘ambiguous’ identification. When all matches within the threshold are different species to the query, the result is scored as ‘false’ (similar to ‘incorrect’ in the bestCloseMatch method).

**Barcode test of species delimitation**

Apart from investigating the potential of DNA markers in identifying species, we explored their ability in assigning morphologically delimited species into genetic units, i.e. ‘MOTUs’ or ‘genetic species’ (sensu Saunders and McDevit 2013). We considered MOTUs as groupings or clusters of specimens that fall around a medoid. The goal is to verify the optimal number of clusters (species) that may be inferred from the pairwise genetic distance matrices of Psoraleoid legumes. A match between our genetic species and morphologically delimited species would indicate that one could serve as a surrogate for the other (see Stahlhut et al. 2013), and thus lend support to the discriminatory power of DNA barcoding. We used partition around medoids (PAM) approach using the R package Cluster (Mächler et al. 2015; R Core Team 2015). Our decision in choosing PAM was made after testing the performance of several clustering algorithms including ‘Agglomerative Nesting (agnes)’, ‘Divisive Analysis Clustering (diana)’ and ‘Fuzzy Analysis Clustering (fanny)’. Results from these other approaches were not reported for at least one of the two main reasons. Firstly, they yielded identical results to PAM and are less straightforward to explain. For example, fanny does not produce unique clusters. Instead, it groups each species (probabilistically) to multiple clusters. The second reason was that the methodologies employed by some of the algorithms do not easily accommodate the restriction of cluster sizes.

The PAM algorithm works as follows: given a specific number of clusters (k), desired from a distance matrix, PAM searches for species (here referred to as medoids) that are representative of the data. The number of medoids sought is usually the same as the number of desired clusters k. Each cluster is then constructed such that the distance of any other sample, in the cluster, from its medoid is minimal. Cluster sizes between 2 and 69 were first investigated for each distance matrix. An optimal cluster size was then chosen as the one that yielded the maximum silhouette coefficient (Kaufman and Rousseeuw 1990). A silhouette coefficient measures the quality of clustering, derived as an average of the

<table>
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<th>Taxon name</th>
<th>Collector</th>
<th>Number</th>
<th>BOLD ID</th>
<th>Herbarium</th>
<th>Distribution</th>
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*Table 1. Continued*
silhouette widths over all species. We used the silhouette width as an aggregate of a measure of the suitability of a cluster for each observation it contains relative to the next best cluster for the observations. Silhouette coefficients range between 0 and 1.

**Barcode test for phylogenetic signal**

We explored the potential of the DNA barcode data to reveal microevolutionary patterns by testing for phylogenetic signal in the affinity of lineages to fire-survival and regeneration strategies. We used a phylogeny of the southern African Psoraleoid species and a binary matrix of reseeders versus resprouters. The phylogeny was reconstructed using a combination of matK and *rbcLa* data sets, based on a maximum-likelihood (ML) approach (Stamatakis *et al*. 2008), enforcing topological constraints from a consensus tree of the Bayesian analysis of the data set. We used the GTR + G + I substitution model based on the result of Akaike information criterion from Modeltest v.2.3 (Nylander 2004), and ran 1000 ML searches. Phylogenetic signal was tested on the ML best tree and binary matrix of reseeders versus resprouters using the *D* statistics of Fritz and Purvis (2010) in the R package Caper (Orme *et al*. 2012). The *D* statistics calculates the sum of changes of a binary trait along the branches of a phylogeny, and compares it with a random model and clumping expected under a Brownian evolution. Significance was assessed by shuffling the trait values 999 times at the tips of the phylogeny. *D* = 1 corresponds to a random distribution of traits at the tip of the phylogeny; *D* = 0 corresponds to a Brownian motion model (Fritz and Purvis 2010).

**Results**

For the core barcode loci, we obtained 332 sequences (165 and 167 for *matK* and *rbcLa*, respectively) from 172 species representing 72 *Otholobium* and 100 *Psoralea*. Sequence recoverability was higher for *rbcLa* than for *matK* (98.1 and 97.1 % of specimens, respectively, Fig. 4). The combined *matK* + *rbcLa* sequence data were obtained from 98.1 % of the specimens sampled (Fig. 4). For *rbcLa*, we recovered 95.7 % of the 69 species sequenced, and 93.6 % for *matK* and when combined with *rbcLa*, i.e. *matK* + *rbcLa*. Both barcodes combined yielded a total of 1326 bp (770 bp for *matK* and 549 bp for *rbcLa*).

The mean interspecific distances for the single and combined regions are lower than 1 %, ranging from 0.002013 in *rbcLa* to 0.008612 in *matK*. The mean intraspecific variation for each and combined DNA regions was also low, ranging from 0.000108 in *rbcLa* to 0.001251 in the combined data set, *matK* + *rbcLa*. The mean intraspecific distances in all cases are significantly lower than interspecific distances (Wilcoxon test, *P* < 0.0001). The minimum interspecific genetic distance is greater than the maximum intraspecific genetic distance in *matK* + *rbcLa* data set (Fig. 5A), indicating the existence of a barcode gap in the data set. The comparison between the lowest interspecific distances (red lines) versus the maximum intraspecific distances (black lines) is shown in Fig. 5B. Further, we found 72 % (116) of the individuals with barcode gap and 28 % (45) without a barcode gap in *matK* + *rbcLa* data set. We also found 12 % (19) of the individuals with barcode gap and 88 % (152) without a barcode gap in *matK* data set. Lastly, we found only 3 % (2) of the individuals with barcode gap in *rbcLa* data set and 97 % (168) without a barcode gap.

Testing the efficacy of DNA barcoding based on discriminatory potential shows that the calculated thresholds ranged from 0.045 in *matK* to an optimized value of 0.36 for the full data set (*matK* + *rbcLa*). Using these cut-offs, we found 100 % true and correct identification in all the data sets for the near-neighbour and best close match analyses, respectively, identifying the individuals to their respective genera (*Psoralea* or *Otholobium*). In terms of identifying the individuals at the species level, we found 25 % success rate for *matK* compared with 4 % in *rbcLa* for the near-neighbour method, which did not improve when the two barcodes were combined (*matK* + *rbcLa*) (Table 2). Similarly, for the best close match analysis, *matK* + *rbcLa* and *matK* exhibited 11 % correct identification rate as opposed to failure in *rbcLa* (0 %) data set (Table 2).

Of the 69 morphologically delimited species included in the analyses, varying discriminatory power in the performance of the DNA markers in grouping specimens into generic species (MOTUs) was found. *rbcLa* grouped all the specimens into 7 genetic species only (silhouette

![Figure 4](https://example.com/figure4.png)
coefficient = 0.98), followed by matK (33 genetic species; silhouette coefficient = 0.84; Table 3). The combination of matK + rbcLa grouped specimens into 37 genetic species unit (silhouette coefficient = 0.84). We, therefore, discussed our results based on the core barcode, i.e. matK + rbcLa data set.

Lastly, we found a weak but significant phylogenetic signal in the affinity of lineages to fire-survival and regeneration strategies. This was significant under the Brownian motion model ($D_{\text{resprouters}} = 0.797, P = 0.003$ and $D_{\text{reseeders}} = 0.798, P = 0.002$, where $D = 0$ corresponds to a Brownian motion model, and $D = 1$ indicates no

Table 2. Performance of the DNA barcodes in identification of individuals to species or genera of Psoraleoid legumes evaluated based on discriminatory potential. Values in parenthesis represent identification of individuals to genera. ‘True’ indicates instances when the near-neighbour method finds the closest individual in the data set and their names are the same or ‘False’ if different. ‘Correct’, ‘Incorrect’, ‘Ambiguous’ and ‘No id’ are used in the best close match method, when the name of the closest match is the same, different, more than one species is the closest match and no species are within the threshold distance, respectively.

<table>
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<tr>
<th>DNA barcoding regions</th>
<th>Number of genetic species (MOTUs)</th>
<th>Near neighbour</th>
<th>Best close match</th>
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<tr>
<td></td>
<td></td>
<td>True (%)</td>
<td>False (%)</td>
</tr>
<tr>
<td>matK + rbcLa</td>
<td>36</td>
<td>25 (100)</td>
<td>75 (0)</td>
</tr>
<tr>
<td>matK</td>
<td>33</td>
<td>25 (100)</td>
<td>75 (0)</td>
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<tr>
<td>rbcLa</td>
<td>7</td>
<td>4 (100)</td>
<td>96 (0)</td>
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Table 3. Genetic species delimited using the best DNA barcode region (matK + rbcLa) identified in this study.

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<td>6</td>
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Continued
phylogenetic signal) (Fig. 6). Multiple origin of reseeder habit is observed in both genera, but it is predominant in *Psoralea* (Fig. 6).

**Discussion**

A key criterion for a standard plant barcode is universality, meaning that the DNA barcode should be easily recovered from all plants, ideally with a single primer pair (CBOL 2009). Our amplification and sequencing success was higher for *rbcLa* than for *matK*, consistent with the results of several other studies that sampled broadly across land plants (e.g. Lahaye et al. 2008; CBOL 2009; Xiang et al. 2011a; Saarela et al. 2013). Recovery of *rbcLa* was higher (98.1 %) than *matK* in this study. This corresponds to the results of other studies on plants in which *rbcLa* recovery ranged from 90 to 100 % (Fazekas et al. 2008; Lahaye et al. 2008; CBOL 2009; Jeanson et al. 2011; Pang et al. 2011; Xiang et al. 2011a; Kuzmina et al. 2012; Saarela et al. 2013).

Several other criteria have also been defined for the identification of the best DNA barcode marker (Hebert et al. 2004a; Kress and Erickson 2007; Lahaye et al. 2008; CBOL 2009). Firstly, it should exhibit a barcode gap, i.e. higher genetic variation between species than within species (Meyer and Paulay 2005). Secondly, it must provide a maximal discrimination among species. We measured the efficacy of the core plant DNA barcode regions (*matK* and *rbcLa*) (CBOL 2009) to identify African *Psoraleoid* legumes using the two approaches: ‘barcode gap’ and discriminatory potential (Meyer and Paulay 2005). We found that interspecific distance is significantly

### Table 3. Continued

<table>
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<tr>
<th>No.</th>
<th>Composition of genetic species or MOTUs</th>
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<td>[1] <em>P. verrucosa</em> Muasya &amp; Stirton3270</td>
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Figure 6. Maximum-likelihood tree of Psoraleoid legumes derived from a combination of the core DNA barcodes matK and rbcLa showing the distribution of fire-survival and regeneration strategies as reseeders (red) versus resprouters (blue).
greater than intraspecific distance. Our mean distances correspond to the results obtained in other plant groups such as Myristicaceae (Newmaster et al. 2008), Rosaceae (Pang et al. 2011), Taxus L. (Taxaceae) (Liu et al. 2011) and in regional Canadian Arctic Flora (Saarela et al. 2013). The second approach was that of Meier et al. (2006), i.e. comparing the smallest interspecific versus the greatest intraspecific distances, instead of comparing the mean distances alone. This approach also reveals the existence of a barcode gap, thus confirming the barcode potential of all the candidates. However, the combination of matK and rbcLa data sets (matK + rbcLa) in all the cases showed greater intraspecific variation than the individual regions alone. This supports the recommendation of the CBOL (2009) that a combination of the two regions (matK and rbcLa) is the preferred standard barcode region for plants.

In addition, we found that all the three data sets have a strong discriminatory power (100 %) in identifying individuals to their respective genera within the Psoraleoid legumes using the near-neighbour and the best close match methods. This supports the utility of DNA barcoding as a means to identify and allocate species between the two genera. Multiple other studies have demonstrated that the core barcode loci routinely provide high discrimination at the genus level, usually >90 % (e.g. Kress et al. 2009; Saarela et al. 2013). Accordingly, we found that rbcLa and matK loci singly distinguish 100 % of genera in our data set. However, their application within species yielded a poor discrimination success, i.e. <50 % with more proportion of ambiguity (51 % matK + rbcLa data set to 79 % in rbcLa data set; Table 2). This result is not surprising, given that several other plant studies have reported poor utility of the core DNA barcodes at lower taxonomic level especially among closely related species and in taxa characterized by recent rapid radiation and hybridization. For example, Clement and Donoghue (2012) reported low levels of discrimination and genetic variation among closely related species of Viburnum. Similarly, Xiang et al. (2011b) reported that rbcLa alone was unable to distinguish genera within Juglandaceae, and neither rbcLa nor matK could discriminate species of Berberis, Ficus or Gossypium (Piredda et al. 2011). In taxa with hybridization issues, for example, Quercus, matK and rbcLa were unable to distinguish any of the 12 sympatric species examined (Roy et al. 2010). The possible causes of the poor discrimination of the species in Psoraleoid legumes observed in this study can be attributed to their recent rapid radiation (Egan and Crandall 2008) and multiple instances of strong hybridization (A. Bello, C.H. Stirton, S.B.M. Chimphango, A.M. Muasya, in preparation; see examples in paragraph below) among the species. Given these caveats, it is clear that additional variable loci are necessary to improve the within-species discrimination success as recommended by the CBOL (2009).

Another feature of interest is the low congruence in assigning morphologically delimited species to genetic species. Several reasons could account for this. Firstly, it could suggest that species are generally not monophyletic (Rieseberg and Brouillet 1994). Secondly, the mis-match could be due to poor performance of the DNA barcodes resulting in over-splitting of taxa. Thirdly, it could be that speciation events do not always match morphological differences, indicating that rapid changes in morphology can occur with minimal evolutionary change (Adams et al. 2002). Fourthly, it could indicate that taxa whose multiple accessions are appearing in diverse clades represent cryptic species, where broad morphological concepts on species are masking genetic patterns. This may be true in Otholobium where widespread species (O. candidans, O. striatum and O. hirtum) may be treated too broadly. Hybridization may account for some of the patterns in Psoralea as some of the taxa have been observed forming hybrids in the field, e.g. P. pinnata × P. aculeata, P. sordida × P. forbesii and P. intonsa × P. oreopola.

In general, there was a weak but significant phylogenetic signal in fire-survival and regeneration strategies of lineages as reseeders or resprouters in Psoraleoid legumes than would be expected by chance. Lineages show significant phylogenetic conservatism in their affinity to fire-survival and regeneration strategies with more clustering of resprouters at the tip of the phylogeny than might be expected by chance. Our phylogeny suggests a multiple origin of these traits implying that the species inherited the resprouting trait from their most recent common ancestor. We hypothesize that the scattering of the reseeding trait across the phylogenetic tree was the result of independent evolutionary events (convergent evolution), probably as a response to fire. It could also mean that the character was inherited from a more ‘basal’ ancestor of the group and then ‘switched off’ in some species but not in others again, in response to fire. However, this remains hypothetical at this stage, pending the availability of more data.

Legumes are regarded as one of the most successful families of flowering plants on Earth both from evolutionary and ecological perspectives, owing to their flexible adaptation to different environments (Rundel 1989). This is evident in the way resprouters and reseeders have evolved to survive in their respective microhabitats in the CFR (Schutte et al. 1995), and frequently dominant in after-fire landscapes. Previous comparative studies on these functional groups have focussed on aspects of taxonomy and physiology (Schutte et al. 1995; Power et al. 2011). Here, we provide evidence of a weak but significant
phylogenetic signal in fire response trait of lineages as reseeders or resprouters in Psoraleoid legumes than expected by chance. Schutte et al. (1995) suggested that there is a substantial difference between resprouters and reseeders, adding that gene flow between resprouting parents and their offspring may occur over time, since the parents are not killed by fire. Seed set does occur in resprouters but is generally very poor and may not occur over a number of fire episodes. The seeds of resprouters are generally larger than those produced copiously by all reseeders (C. H. Stirton, pers. obs.).

In contrast, temporal isolation in gene flow might occur in reseeding taxa, as there is less chance of interbreeding between parents and offspring, and thus, each new generation may be a cohort of its own. It is not known how much seed remains in the seed bank and it is possible that some seeds may germinate in a later fire episode. It should be borne in mind, however, that parents and offspring could coexist if fires are patchy, if fire temperature affects the proportion of the seed bank that can be stimulated to germinate, if fires are too hot and if the seed bank comprises different genetic cohorts. The consequence of these is that speciation would more readily occur in reseeders, as interbreeding between parents and their progeny is unlikely. Given these caveats, our results provide some extrinsic support for the idea that reseeders speciate faster than resprouters as the number of reseeding species in our study outnumbers that of the resprouters. Schutte et al. (1995) reported that there is a faster rate of speciation and differentiation within reseeders, than in resprouters, but did not provide any genetic evidence for this. Most reseeding species of legumes in the CFR are short lived (ca. 8 – 15 years), with few exceptions, e.g. in Podalyria calyptrata and in some forest margin species of Virgilia with relatively long life spans (>40 years). In the younger genus Psoralea, there are more reseeders than resprouters, whereas in the older genus Otholobium, there are more resprouters than reseeders and fewer species overall. Among the Psoraleoid legumes, reseeders are frequently observed on wet valleys near mountain streams, while resprouters are common in drier habitats, a phenomenon also observed in African Restionaceae, which shares increased diversification in reseeders (Litsios et al. 2014).

Conclusions
This study showed that DNA barcoding may be useful in species identification and in inferring the impacts of recurrent fires on gene flow in resprouting and reseeding taxa in the CFR. In general, we showed that Psoraleoid legumes of the CFR exhibit a barcoding gap with high scores for correct identification of individuals to their respective genera. We found a considerable match between genetic and morphologically delimited species supporting the discriminatory power of DNA barcoding. We also found that lineages in Psoraleeae showed a weak but significant phylogenetic conservatism in their affinity for different fire response trait with more clustering of resprouters in Psoralea at the tip of the phylogeny than expected by chance. Our phylogeny suggests a convergent origin of the reseeding trait in African Psoraleoid genera. We conclude that these novel microevolutionary patterns might be acting continuously over time to produce multi-scale regularities of biodiversity especially in a biodiversity hotspot as the CFR.

Accession Numbers
All data for the project were managed in the BOLD database in a project called ‘Fabaceae@UCT’ (project code FAUCT). Detailed voucher information, including the scientific names of taxa sampled, BOLD ID numbers, collectors and collection numbers, for all sequences are given in Table 1.

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Contributions by the Authors
A.B. and B.H.D. performed the data analyses and were involved in writing and editing; C.H.S., A.M.M., S.B.M.C. and A.B. performed the fieldwork and were involved in writing and editing; M.v.d.B. and O.M. provided contribution to the concept and the design of the work and also handled the sequencing activities. All the authors read and approved the final manuscript.

Conflict of Interest Statement
None declared.

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**Literature Cited**


