

Massively parallel sequencing and analysis of expressed sequence tags in a successful invasive plant

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• **Background** Invasive species pose a significant threat to global economies, agriculture and biodiversity. Despite progress towards understanding the ecological factors associated with plant invasions, limited genomic resources have made it difficult to elucidate the evolutionary and genetic factors responsible for invasiveness. This study presents the first expressed sequence tag (EST) collection for *Senecio madagascariensis*, a globally invasive plant species.

• **Methods** We used pyrosequencing of one normalized and two subtractive libraries, derived from one native and one invasive population, to generate an EST collection. ESTs were assembled into contigs, annotated by BLAST comparison with the NCBI non-redundant protein database and assigned gene ontology (GO) terms from the Plant GO Slim ontologies.

• **Key Results** Assembly of the 221 746 sequence reads resulted in 12 442 contigs. Over 50 % (6183) of 12 442 contigs showed significant homology to proteins in the NCBI database, representing approx. 4800 independent transcripts. The molecular transducer GO term was significantly over-represented in the native (South African) subtractive library compared with the invasive (Australian) library. Based on NCBI BLAST hits and literature searches, 40 % of the molecular transducer genes identified in the South African subtractive library are likely to be involved in response to biotic stimuli, such as fungal, bacterial and viral pathogens.

• **Conclusions** This EST collection is the first representation of the *S. madagascariensis* transcriptome and provides an important resource for the discovery of candidate genes associated with plant invasiveness. The over-representation of molecular transducer genes associated with defence responses in the native subtractive library provides preliminary support for aspects of the enemy release and evolution of increased competitive ability hypotheses in this successful invasive. This study highlights the contribution of next-generation sequencing to better understanding the molecular mechanisms underlying ecological hypotheses that are important in successful plant invasions.

Key words: ESTs, genomics, invasive species, maternal effects, rapid adaptation, selection, *Senecio madagascariensis*.

INTRODUCTION

Invasive plant species pose a significant threat to global economies, agriculture and biodiversity (Richardson and Pysek, 2006; Thuiller *et al.*, 2006; Prentis *et al.*, 2007), and they are recognized as a major component of human-induced global change (Wilson *et al.*, 2009a). As international trade and travel continue to grow (Wilson *et al.*, 2009a), it seems likely that alien plant invasions will continue to increase at a rapid rate. Understanding the process of invasion is critical to the management of invasive species and for identifying species that may pose a risk in the future. Despite progress towards understanding the ecology of plant invasions (Thuiller *et al.*, 2006; Milton *et al.*, 2007; Wilson *et al.*, 2007, 2009b; Proches *et al.*, 2008), relatively little headway has been made in

identifying the evolutionary or genetic factors responsible (Sakai *et al.*, 2001; Lee, 2002; Prentis *et al.*, 2008).

Evolutionary changes in introduced populations were first recognized as an important process in biological invasions over 35 years ago (Baker, 1974). However, the majority of evolutionary research in invasion biology has been conducted within the past 10 years, and has largely concentrated on comparing genetic diversity between source and invasive populations (Bossdorf *et al.*, 2005; Kang *et al.*, 2007; Dlugosch and Parker, 2008; Prentis *et al.*, 2009). Fewer studies have examined the role of adaptive evolution in biological invasion (Whitney *et al.*, 2006; Dlugosch and Parker, 2008), and even fewer studies have attempted to identify the source of genetic and epigenetic variation underlying adaptation in an invasive species (Parisod *et al.*, 2009).

Two hypotheses commonly invoked to explain the success of invasive species are the enemy release hypothesis (ERH) and the evolution of increased competitive ability (EICA). These hypotheses theorize that invasive species will escape the negative effects of their natural enemies in the introduced range (Blossey and Notzold, 1995; Keane and Crawley, 2002; Colautti *et al.*, 2004; Orians and Ward, 2010), but they are supported in only approx. 50 % of studies (Bossdorf *et al.*, 2005). Although these arguments are intuitive, the extent for evolution to occur following release from enemies in the introduced range depends on the extent to which natural enemies controlled the abundance of these species in the native range. For species that do escape the negative effects of their natural enemies in the introduced range, EICA predicts that natural selection will favour those genotypes with decreased allocation to defence and increased allocation to competitiveness. Although these hypotheses have been extensively tested ecologically, little research has investigated the underlying molecular mechanisms.

Research in this area has lagged largely because the kind of genomic or expressed sequence tag (EST) resources required to identify candidate genes or genomic changes associated with invasiveness are not yet developed for most invasive species. In fact, only few invasive species have had significant genomic resources developed for this purpose (Lai *et al.*, 2006; Broz *et al.*, 2007, 2009; Barker *et al.*, 2008). Although genomic tools developed for crop or model plants such as rice or *Arabidopsis* may be applied to their weedy relatives, genomic resources must be developed specifically for the invasive species to best identify the genetic changes responsible for success in recipient environments and to manage invasive populations. For most non-model species, EST data have been traditionally obtained through generation of normalized complementary DNA (cDNA) libraries and Sanger sequencing (Bouck and Vision, 2007). However, next-generation sequencing approaches, such as massively parallel 454 pyrosequencing, can improve this process (Margulies *et al.*, 2005; Cheung *et al.*, 2006; Goldberg *et al.*, 2006; Moore *et al.*, 2006; Wicker *et al.*, 2006; Huse *et al.*, 2007) and have recently been used to rapidly and accurately characterize a transcriptome sequence in a non-model organism (Vera *et al.*, 2008). Therefore, next-generation sequencing can be an effective method for developing large-scale genomic resources for non-model invasive organisms.

Senecio madagascariensis, a native of southern Africa, is a highly invasive plant that has been introduced to Australia, Japan, Argentina, Mexico and Hawaii (Radford *et al.*, 2000; Le Roux *et al.*, 2006; Lopez *et al.*, 2008). Its polyploid form, *Senecio inaequidens*, is invasive throughout southern Europe (Lafuma *et al.*, 2003; Cano *et al.*, 2007; Lafuma and Maurice, 2007; Bossdorf *et al.*, 2008; Monty *et al.*, 2009; Monty and Mahy, 2009; Lachmuth *et al.*, 2010). In Australia alone, annual losses of up to US\$12 million have been directly linked to fireweed populations (Le Roux *et al.*, 2006). Fireweed often colonizes disturbed habitats or overgrazed pastures throughout its introduced range where it competes strongly with native species and pasture plants for light, moisture and soil nutrients.

Recent molecular investigations have shown that Australian and Hawaiian fireweed populations are of South African origin

(Scott *et al.*, 1998; Le Roux *et al.*, 2006), but were probably introduced to Australia from multiple sources in South Africa (Radford *et al.*, 2000). Multiple introductions of genetically differentiated source populations provide opportunities for admixture in the introduced range, which has been proposed as a stimulus for adaptive evolution and invasiveness in many introduced plants (Ellstrand and Schierenbeck, 2000). Investigations of neutral genetic variation have found similar levels of genetic diversity in invasive Australian (E. E. Dormontt *et al.*, University of Adelaide, Australia, unpubl. res.) and Hawaiian (Le Roux *et al.*, 2010) populations compared with source populations from South Africa, indicating that this species has not gone through a strong bottleneck during population founding. High levels of neutral genetic variation in introduced populations (Le Roux *et al.*, 2010) indicate that substantial standing genetic variation at loci underlying important traits may also have been introduced. Adaptive evolution in invasive species has been hypothesized to be dominated by alleles from standing genetic variation (Prentis *et al.*, 2008), because favourable alleles are immediately available and usually occur at a greater frequency in populations than do neutral or deleterious alleles (Barrett and Schluter, 2008). However, it remains unclear whether admixture or adaptation from standing genetic variation was responsible for the successful invasion of *S. madagascariensis*.

As a first step towards understanding the molecular basis for adaptive evolution in invasive species, we have generated one normalized and two subtractive EST libraries representing one native South African and one invasive Australian population of *S. madagascariensis*. Here we describe the annotation of our *S. madagascariensis* ESTs by sequence comparison with the entire core protein database of NCBI. We also provide an initial list of candidate genes from our subtractive libraries that could be utilized in future experiments to test for correlations between patterns of gene expression and ecological or evolutionary factors that may be associated with successful invasions. In addition, we mined singlet contigs for microsatellites to be utilized in genome scan experiments to examine contemporary selection during the invasion of *S. madagascariensis*.

MATERIALS AND METHODS

Plant material

Senecio madagascariensis Poir. (fireweed) seeds were obtained from one native South African and one invasive Australian population. The South African population was collected from East London (33°02'S, 27°50'E), while seeds from the Australian population were collected from Springbrook National Park (28°08'21"S, 153°16'41"E). The Springbrook population was selected because it was an allopatric site identified by Prentis *et al.* (2007). This site contained no hybrid plants or seed and was isolated from any *Senecio pinnatifolius* population by more than 10 km. The seeds were placed in Petri dishes containing germination paper moistened with distilled water. Germination took place in a growth cabinet under standard culture conditions (12/12-h light–dark photoperiod at a constant temperature of 20 °C). Seedlings with fully expanded cotyledons were transferred and planted in 8-cm pots in California mix and returned to standard culture conditions in the growth cabinet.

BLASTx hits. Contigs were clustered if they shared at least one ‘good hit’, defined as the best BLASTx hit and any subsequent hit with an *E*-value at least 99 % as good as that of the best hit. We refer to groups of clustered contigs as ‘clusters’ and to contigs that did not cluster with any other contig as ‘singlets’. Annotations were transferred from best BLASTx hits to each cluster or singlet.

Gene ontology (GO) terms from the Plant GO Slim (<http://www.geneontology.org/GO.slims.shtml>) ontologies were assigned to each contig using Blast2GO with default settings (Conesa *et al.*, 2005). We then used WEGO (Ye *et al.*, 2006) to determine the numbers of contigs annotated with each GO term for each library. We tested for significant enrichment of GO terms in different libraries using Fisher’s exact tests, corrected for multiple tests using the false discovery rate as implemented in the R package *fdrtool* (Strimmer, 2008; R Team, 2009).

Microsatellite mining

We mined singlet contigs for microsatellites, using SSRIT (Temnykh *et al.*, 2001; <http://www.gramene.org/db/searches/ssrtool>), which is a Perl script that identifies all microsatellite markers (simple sequence repeats, SSRs) within a set of sequences. We set the script to identify all possible di-, tri- and tetranucleotide repeats with a minimum of five subunits. Although some groups have used higher cut-offs (Kantety *et al.*, 2002), our method of relaxing the threshold maximizes SSR discovery while still producing a high percentage of polymorphic loci (Pashley *et al.*, 2006).

RESULTS

454 EST sequencing and contig assembly

From a single Roche 454 GS FLX run, a total of 221 746 EST sequences that exceeded our minimum quality thresholds were generated from four cDNA libraries constructed from various tissue types, growth stages and treatments (Table 1). The average size of these ESTs was 210 bp, generating 46.57 Mbp of sequence. The two subtractive libraries (Libraries 3 and 4) had a much greater quantity of ESTs that met our quality thresholds than either of the normalized libraries (Libraries 1 and 2; Table 2). All EST sequences were deposited in the short read archive of the NCBI database (short read archive ID for Library 1, SRX001877; Library 2, SRX001878; Library 3, SRX001880; Library 4, SRX001879) or can be obtained via email from the corresponding author. Assembly of high-quality ESTs formed 12 442 contig sequences of average length 264 bp, with an average depth of coverage of 5.68 sequences per nucleotide position. Some highly expressed contigs were sequenced in over 1000 reads. Overall our average contig length was longer (264 bp) and our average coverage substantially higher (5.67-fold) than most previous studies using 454 sequencing in non-model species: 197 bp at 2.3× coverage (*Melitaea cinxia*; Vera *et al.*, 2008) and 245 bp (*Eucalyptus grandis*; Novaes *et al.*, 2008).

TABLE 2. Assembly statistics of the *Senecio madagascariensis* ESTs, including number of ESTs, number of large contigs, mean contig size, largest contig and total contig for the four libraries separately and all libraries combined

Assembly statistics	Library 1	Library 2	Library 3	Library 4	All libraries
Number of ESTs	36 856	26 896	76 661	81 333	221 746
No. of large contigs (≥ 500 bp)	156	71	81	277	804
Mean contig size (bp)	236	227	254	262	264
Largest contig	1210	822	1028	1129	1575
Total contigs	3786	2859	2388	5769	12 442

Library 1, sonicated normalized library; Library 2, restriction enzyme digested normalized library; Library 3, South African subtractive library; Library 4, Australian subtractive library; All Libraries, assembly statistics for all libraries combined.

TABLE 3. BLASTx analysis statistics of the *Senecio madagascariensis* EST libraries against the NCBI NR database

Library	Total contigs	Singlet contigs	Clustered contigs	No. of clusters	No-hit contigs
1	3786	1389	265	124	2132
2	2859	912	158	75	1789
3	2388	754	430	168	1204
4	5769	2169	1134	437	2466
Total	12442	3946	2237	872	6259

Library 1, sonicated normalized library; Library 2, restriction enzyme digested normalized library; Library 3, South African subtractive library; Library 4, Australian subtractive library.

Functional annotation of contigs

Among the 12 442 contigs, 6183 (49.7 %) had hits exceeding our significance threshold (*E*-value < 1×10^{-5}) in the NR database (Table 3). Of the 6183 contigs that had a significant hit in the NR database, 3946 were singlets and 2237 formed 872 clusters of contigs (contigs sharing best or near-best BLAST hits). The number of contigs that formed a cluster ranged from two to 22, with an average of 2.4 ESTs per cluster.

Overall, the contigs were quite diverse in the types of transcripts present, based on the number of GO terms assigned to contigs (Fig. 1). Given the diversity in GO terms assigned, it appears we were successful in capturing a number of functional gene categories in our libraries.

To investigate which categories of genes are over-represented in the subtractive libraries, we tested for significant enrichment of GO terms in Libraries 3 and 4 relative to contigs assembled from all libraries, and also between Libraries 3 and 4. No GO terms were over-represented in subtractive libraries relative to the normalized library after correction for multiple tests. The molecular transducer activity GO term was significantly ($P < 0.001$) enriched in Library 4 (South African population) compared with Library 3 (Australian population) after correction for multiple tests (Fig. 1B; see Table 4 for molecular transducer gene list). We undertook a literature search of the genes and gene families identified with the molecular transducer activity GO term from the South African library. Over 40 % of these genes respond to biotic stimuli and have

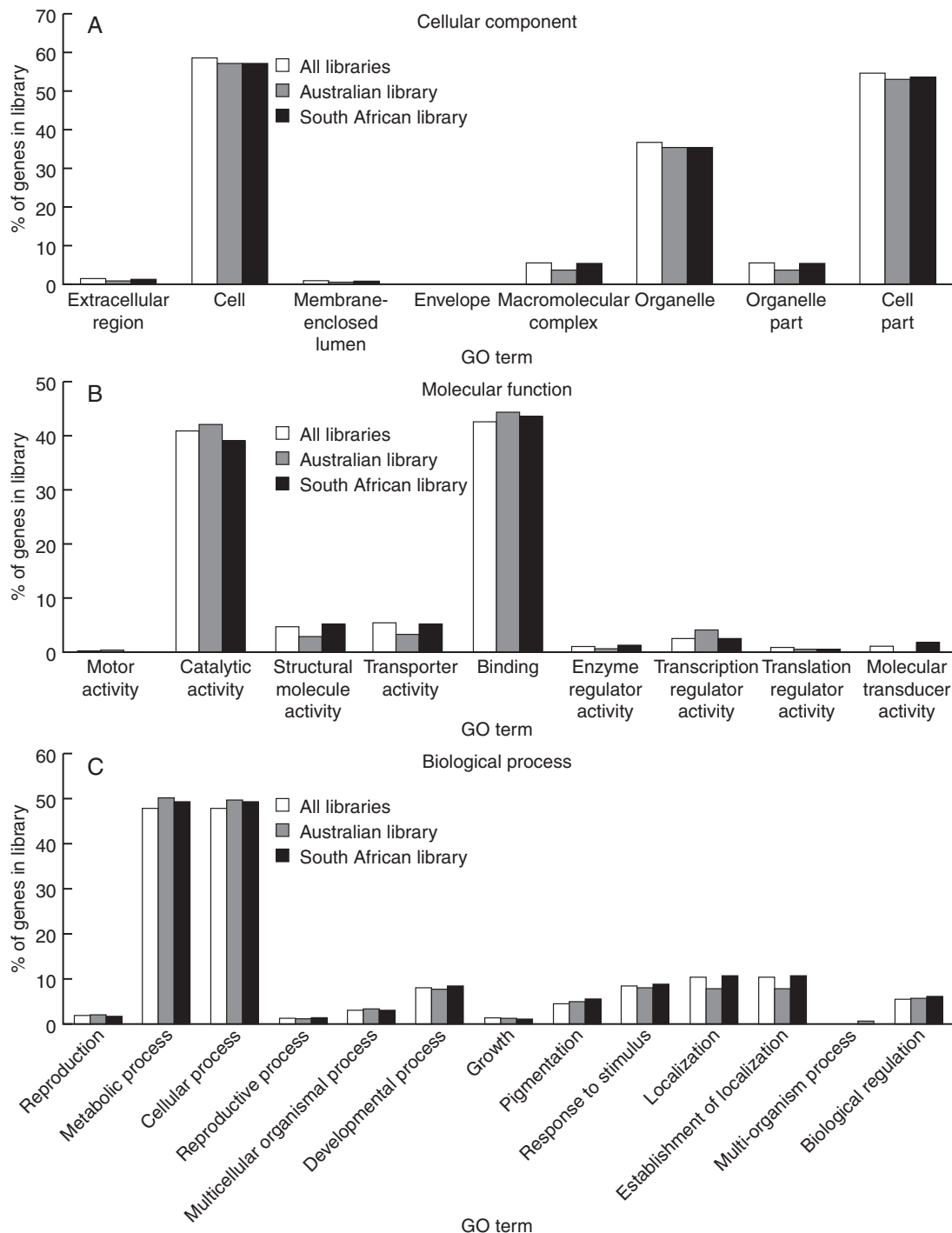


FIG. 1. Proportional representation of GO terms in the Australian, South African and total EST libraries for (A) cellular component, (B) molecular function and (C) biological process domains.

been implicated in defence response to viral, fungal and bacterial pathogens.

Microsatellite markers for mapping and population studies

A total of 190 SSRs from 185 different contigs were detected in the 3946 singlet contigs. This produced an overall SSR discovery rate of 4.8%, or one microsatellite in every 20 singlet

contigs. The most abundant repeat motifs were di-nucleotide repeats (101), closely followed by tri-nucleotide repeats (86) but very few tetra-nucleotide repeats (three).

DISCUSSION

Here we have described an EST collection generated by 454 pyrosequencing and *de novo* assembly to characterize

TABLE 4. Molecular transducer GO class genes that were over-represented in the native range EST library compared with the invasive range EST library

Gene	Contig ID	Length (bp)
Phototropic-responsive NPH3 family protein	contig00533; contig01103; contig02636; contig02080	294; 241; 243; 156
Histidine phosphotransfer protein	contig00734	211
Pyruvate dehydrogenase kinase	contig00887	234
Ethylene insensitive 4	contig01144	241
Root phototropism protein	contig03698	240
Guanine nucleotide-binding protein beta	contig03914	241
Phototropin B1 blue light photoreceptor	contig03963; contig05165	240; 164
PAC motif-containing protein	contig04546; contig04557	370; 474
Vacuolar sorting receptor protein	contig00082	200
Somatic embryogenesis receptor kinase 1	contig00379; contig00577; contig05630; contig05684	356; 439; 586; 172
Histidine kinase 3	contig00384; contig01470; contig01600	548; 242; 485
Eukaryotic translation initiation factor 3	contig00424; contig01524	704; 178
ER lumen retaining receptor	contig01095; contig01221; contig02806	241; 237; 404
LrgB-like family protein	contig01889	241
Somatic embryogenesis receptor-like kinase2	contig02285	241
Phytosulfokine receptor precursor	contig03383	241
Interleukin-1 receptor-associated kinase	contig03394	186
Transmembrane BAX inhibitor motif-containing protein	contig03442	241
Leaf senescence related protein	contig04299	206
Signal recognition particle receptor protein	contig05496	228

'Gene' is the best BLAST hit in the NCBI database with an *E*-value cut-off of 1×10^{-5} . 'Contig ID' is the number assigned to each contig in our database and 'length' is the length of individual contigs.

the transcriptome of a non-model invasive species, *S. madagascariensis*. The assembly of long contigs with an average length of 264 bp allowed us to achieve high annotation success for species with a lack of genomic resources. Sequencing of two subtractive libraries and a single normalized library also allowed initial characterization of gene expression differences between the native and introduced ranges of *S. madagascariensis*. The molecular transducer GO term was significantly over-represented in the native (South African) subtractive library compared with the invasive library. Significantly, many of the genes from this GO term are involved in response to biotic stimulus or are defence response proteins.

Gene annotation and candidate markers for invasiveness

Gene annotation against the NR database in NCBI allowed us to annotate approx. 4800 independent transcripts. From these annotated transcripts we identified a number of genes that may underlie traits of evolutionary and ecological interest. Most notable are genes from the molecular transducer activity GO category that are significantly under-represented in the Australian EST library. Although this result is highly significant, other techniques, such as real-time PCR and large-scale sequence analysis, may be used to provide additional support for this finding. Over 40 % of genes and gene families annotated with the molecular transducer activity term in the South African library respond to biotic stimulus and have been implicated in defence responses to fungal, bacterial and viral pathogens (Thomma *et al.*, 1999; Trusov *et al.*, 2006; Hopkins *et al.*, 2008; Watanabe and Lam, 2008; Zhu *et al.*, 2009).

The under-representation of molecular transducer genes in the invasive range library indicates that they may be associated with important evolutionary responses or maternal effects to natural enemies or other biotic stimuli in invasive populations of *S. madagascariensis*. An under-representation of defence

response proteins in the introduced range provides preliminary support for aspects of the ERH and EICA hypotheses in this successful invasive. It has been hypothesized that differences in the natural enemy community between the native and introduced ranges of invasive plants can promote a range of evolutionary responses in the allocation of resources to plant defence and growth (Blossey and Notzold, 1995; Joshi and Vrieling, 2005; Orians and Ward, 2010). For example, an examination of phytochemicals in the invasive range of wild parsnip has provided evidence for increased production of defence compounds that coincide with the introduction of its specialist herbivore (Zangerl and Berenbaum, 2005). Other recent studies have shown that defence and stress genes are downregulated in invasive populations of sunflower and starthistle (Lai *et al.*, 2008; Stewart *et al.*, 2009). Selection for decreased expression of defence genes in the introduced range of *S. madagascariensis* may explain why genes from the molecular transducer GO category are underrepresented in the Australian library. Alternatively, environmental maternal effects associated with escaping natural enemies in the native range may also explain the under-representation of molecular transducer genes in the Australian library. Both explanations require that introduced populations of *S. madagascariensis* have escaped their specialist natural enemies.

One assumption of the ERH has been supported in the Australian range of *S. madagascariensis* (White *et al.*, 2008a). This study demonstrated that a native herbivore prefers native congeners over the introduced *S. madagascariensis* and that foliage damage was significantly greater on the native species, *S. pinntifolius*, than on *S. madagascariensis* (White *et al.*, 2008a). Although this study fulfils some aspects of the ERH it does not demonstrate that natural enemy damage is reduced in the introduced range compared with the native range. Therefore, other studies should investigate whether the negative impacts of natural enemies are decreased in the introduced range

of *S. madagascariensis*. Furthermore, to confirm whether selection or maternal effects are responsible for the observed pattern of under-representation of molecular transducer genes in the Australian library, candidate genes need to be tested for evidence of recent selection and gene expression differences associated with maternal environments.

EST-derived microsatellite markers

We found 190 EST-associated microsatellites suitable for use as genetic markers in future population-level and mapping studies. The rate of microsatellite discovery in our project (4.8%) is similar to those of most previous studies in plants, which typically range between 2 and 5% when using similar search parameters (Kantety et al., 2002). Many studies have found in the order of 80–90% of EST-derived microsatellite markers to be polymorphic (Bandopadhyay et al., 2004; Ellis et al., 2006; Pashley et al., 2006; Gasic et al., 2009). These microsatellites are likely to be highly transferable across closely related species, as has been found in previous studies (Gutierrez et al., 2005; Pashley et al., 2006; Ellis and Burke, 2007). Therefore, these markers may provide a resource for another highly invasive species, *S. inaequidens* (Lafuma and Maurice, 2007; Bossdorf et al., 2008; Cano et al., 2009; Monty et al., 2009; Monty and Mahy, 2009), and for hybridization studies with the Australian native, *S. pinnatifolius* (Prentis et al., 2007).

Conclusions

Senecio madagascariensis and its closely related congener *S. inaequidens* are highly invasive species that are becoming increasingly used as model species with which to investigate hypotheses in invasion biology (Radford and Cousins, 2000; Lafuma et al., 2003; Cano et al., 2007; Lafuma and Maurice, 2007; Prentis et al., 2007; White et al., 2008a, b, c; Bossdorf et al., 2008; Monty et al., 2009; Monty and Mahy, 2009, 2010). The use of genome scans and gene expression approaches to determine if invasion success may have a molecular genetic basis has been constrained by a lack of genomic resources to identify candidate genes or genomic changes associated with invasiveness. Here, we report an EST collection for *S. madagascariensis* that provides a first step toward understanding the genetic bases of invasion success. Our data provide large-scale EST resources that will further enhance *S. madagascariensis* as a model species for examining hypotheses about invasion success. This resource will aid microarray design and sequencing projects and provides a large number of initial candidate gene markers that can help to answer a number of leading hypotheses in invasion biology.

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