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Rapid evolution or preadaptation in invasive *Jacobaea vulgaris*

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The complete chloroplast genome of 17 individuals of pest species *Jacobaea vulgaris*: SNPs, microsatellites and barcoding markers for population and phylogenetic studies

Running title: Complete chloroplast genome of *Jacobaea vulgaris*

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Abstract

Invasive individuals from the pest species *Jacobaea vulgaris* show different allocation patterns in defence and growth compared to native individuals. To examine if these changes are caused by fast evolution, it is necessary to identify native source populations and compare these with invasive populations. For this purpose we are in need of intraspecific polymorphic markers. We therefore sequenced the complete chloroplast genomes of 12 native and 5 invasive individuals of *J. vulgaris* with next generation sequencing and discovered Single Nucleotide Polymorphisms (SNPs) and microsatellites. This is the first study in which the chloroplast genome of that many individuals within a single species was sequenced. Thirty two SNPs and 34 microsatellite regions were found. For none of the individuals differences were found between the Inverted Repeats. Furthermore, being the first chloroplast genome sequenced in the Senecioneae clade, we compared it with four other members of the Asteraceae family to identify new regions for phylogenetic inference within this clade and also within the Asteraceae family. Five markers (*ndhC-trnV*, *ndhC-atpE*, *rps18-rpl20*, *clpP* and *psbM-trnD*) contained parsimony-informative characters higher than two percent. Finally we compared two procedures of preparing chloroplast DNA for next generation sequencing.

Keywords: phylogenetic markers, *Jacobaea vulgaris*, Asteraceae, cpDNA polymorphisms, inverted repeat

Introduction

Comprising one-tenth of all flowering plants and containing over 20 000 species, the Asteraceae are one of the largest vascular plant families. With the exception of Antarctica, the Asteraceae are distributed on all continents. Species in this family are extremely various in secondary chemistry, inflorescence morphology and chromosome numbers. This huge variation provides great opportunities to acquire insight into the diversification process in this family, which began 42-36 million years ago. The Asteraceae are not only interesting because of their phenotypic and species diversity, but this family also includes members of economically important food crops, herbal species, ornamentals, and plants for the cut flower industry. Other members such as *Jacobaea vulgaris*, *Senecio vulgaris* and *Taraxacum officinale* are weedy and have an economical and ecological impact.

We sequenced the complete chloroplast genome of *J. vulgaris* with next generation sequencing techniques to find new genetic markers that are phylogenetically informative and to discover intraspecific polymorphic markers for population studies. The conservative structure of the chloroplast genome makes it easy to compare with other members of the Asteraceae family. In a recent study of Panero and Funk (2008), 12 major lineages of Asteraceae were found with Bayesian and Maximum Parsimony methods by combining ten chloroplast loci from 108 taxa. Within the subfamily Asteroideae, strong statistical support was found for tribal relationships except for the Senecioneae tribe. In the Bayesian analysis, this tribe was unresolved and in the Maximum Parsimony analysis it was placed as a sister group to Calenduleae without strong statistical support (52% BS). In other studies of Pelsner (2007, 2010) a phylogenetic analysis of the nuclear ribosomal internal spacers (nrITS) and external spacer (ETS) and five chloroplast loci was done to clarify intergeneric relationships within Senecioneae and to delimitate the genus *Senecio*. Although these phylogenies gave more insight, they still lacked strong statistical support and resolution.

No chloroplast genome has been previously sequenced from any species in the Senecioneae clade, and the chloroplast genome sequence of *J. vulgaris* can yield more information about variation within this clade, as well as between clades of the Asteroideae subfamily. In this study the chloroplast genome of *J. vulgaris* (tribe Jacobaea) was compared with *Guizotia abyssinica*, *Helianthus annuus*, *Parthenium argentatum* (all belonging to tribe Heliantheae) and *Lactuca sativa* (tribe Lactuceae). To guide future phylogenetic studies within the Asteraceae family, we identified new phylogenetically informative chloroplast markers by finding differences within and between genome organization.

Jacobaea vulgaris is a troublesome weed that belongs to the Asteraceae family and is native to Europe and western Asia, ranging from Norway through Turkey, and from Great Britain to Siberia. It was first reported in the 1850s in Canada, in 1875 in New Zealand and shortly thereafter in Australia and in 1900 at the west coast of North America. In introduced areas *J. vulgaris* is a pest species, outcompeting local plants and containing pyrrolizidine alkaloids which are toxic to herbivores. Control is difficult, since the lifecycle can vary from annual to short-lived perennial, depending on the genotype. Moreover, seeds remain viable in the soil for several years. *Jacobaea vulgaris* causes four million dollar losses annually to cattle poisoning and control in Australia alone.

Joshi and Vrieling (2005) compared *J. vulgaris* plants from the invasive areas with plants from the native area and found that invasive individuals contained higher pyrrolizidine alkaloid levels, have a 30% higher reproductive effort, are more susceptible to attack by specialist herbivores and less susceptible to generalist herbivores. These results suggest that selection pressures in the invasive area shaped the different allocation patterns in *J. vulgaris* in the invasive areas within 70 generations. However it is possible that introduced populations were derived from native European populations that happened to express pyrrolizidine alkaloid and allocation patterns that are similar to those currently observed in invasive ranges.

To exclude the null hypothesis that these patterns are observed as a result of genetic drift rather than natural selection, native source populations need to be identified and compared to invasive populations. Source populations can be pinpointed by using neutral molecular markers such as AFLPs. A previous study on *J. vulgaris*, based on nuclear AFLP data, did not show a difference in the amount of variation between native and invasive individuals. These findings suggest that introductions from multiple source populations have occurred. Other neutral markers are Single Nucleotide Polymorphisms (SNPs) and microsatellite markers in the chloroplast genome.

Next generation sequencing can produce DNA sequences cheaply and quickly, facilitating the rapid sequencing of nuclear and organellar genomes. Chloroplast genomes are known for their conservative rates of evolution. With an average size of 150 kb, chloroplast genomes are sufficiently large to find differences between and within species. The absence of recombination and maternal transmission of the chloroplast genome (limiting gene flow to seed dispersal only) makes cpDNA markers useful for tracing source population(s).

In this study, we sequenced the chloroplast genome of 17 *J. vulgaris* individuals by using the Illumina Genome analyzer platform. This is the first study sequencing multiple individuals of the same species with next generation sequencing. Multiple individuals were sequenced to reveal intra-specific variation (SNPs and microsatellite loci). Finally, we compared two different procedures of preparation for sequencing the chloroplast genome, namely direct extraction of the chloroplast DNA and amplifying the cpDNA with long range PCR.

Materials and Methods

Extraction of chloroplasts and isolation of DNA from chloroplasts

By using the chloroplast extraction kit of Sigma- Aldrich [CP-ISO] and following the manufacturer's protocol, chloroplasts from sample nr. 17 (see Table 1) were isolated out of 30 g of fresh leaf material. To remove unwanted whole cells and cell wall debris, the blended leaf material with the chloroplast isolation buffer was centrifuged. To separate the intact from the broken chloroplasts a 40 % percoll layer was used. Before DNA extraction, the intact chloroplasts were treated with ST buffer (400 mM sucrose, 50 mM Tris pH 7.8, 0.1% bovine serum albumin) with a final concentration of 25 µg/mL DNase-1 (Sigma Aldrich) per gram of leaf material to digest DNA outside the intact chloroplasts. After

centrifuging, the chloroplast pellet was resuspended in a TEN buffer (100 mM Tris pH 7.2, 50 mM EDTA, 100 mM NaCl, 0.2% β-mercaptoethanol). To extract the DNA from the chloroplasts, the chloroplasts were lysed with 1% sodium dodecyl sulfate followed by a phenol/chloroform step to remove proteins. The DNA was precipitated overnight with 1/10 vol. of 5M ammonium acetate and 1 vol. of isopropanol. After centrifuging, the pellet was washed with 70% ethanol and re-dissolved in TE buffer (1M Tris HCl pH=8.0, 0.5M EDTA).

Table 1. Geographical information, percentage of the chloroplast genome sequenced, method used for preparing the template for Illumina sequencing, lane number on the Illumina platform and reads obtained of the 17 individuals of *Jacobaea vulgaris* that were sequenced.

Sample	Country	Location	Latitude/longitude	% cp genome sequenced	Template sequencing	Illumina lane
1	New Zealand	Haast (South Island)	S 43° E 169°	89.9	Long range PCR	2 (776)
2	Ireland	Caherdaniel	N 51° W 10°	88.5	Long range PCR	2 (545)
3	Norway	Malvik	N 63° E 10°	83.4	Long range PCR	2 (543)
4	Canada	Cardigan	N 46° W 62°	89.8	Long range PCR	2 (838)
5	UK	Padstow	N 50° W 4°	98.3	Long range PCR	2 (1043)
6	Poland	Warsaw	N 52° E 18°	94.3	Long range PCR	2 (650)
7	Spain	Covadonga	N 43° W 04°	91.5	Long range PCR	2 (457)
8	France	Perrogney	N 47° E 05°	89.9	Long range PCR	2 (558)
9	Hungary	Lénárdaróc	N 48° E 20°	86.7	Long range PCR	2 (80)
10	The Netherlands	Ameland	N 53° E 05°	88.6	Long range PCR	2 (468)
11	Australia	Barramonga	S 38° E 143°	90.6	Long range PCR	2 (680)
12	Australia	Franklin (Tasmania)	S 43° E 147°	91.8	Long range PCR	2 (465)
13	UK	Portsmouth	N 50° W 01°	98.9	Long range PCR	2 (1102)
14	Sweden	Kapellskär	N 59° E 53°	99.9	Long range PCR	3 (11 084)
15	New Zealand	Opunake (North Island)	S 39° E 173°	94.7	Long range PCR	2 (691)
16	Germany	Halle	N 51° E 11°	98.7	Long range PCR	2 (805)
17	Spain	Covadonga	N 43° W 04°	99.9	chloroplast DNA extract	1 (18 646)*

Numbers given in parenthesis are the number of single-end reads x 1000. * paired-end reads.

Total DNA extraction

Total DNA extractions from samples 1 to 16 of *J. vulgaris* (Table 1) were carried out on five leaf punches of 1cm diameter each, using the CTAB extraction protocol of Doyle and Doyle (1987).

Long range PCR

To develop primers for long range PCR, the sequences of *Helianthus annuus* (NC007977), *Lactuca sativa* (DQ383816) and *Guizotia abyssinica* (EU549769) were aligned with BioEdit. With the aid of this alignment and the annotation of *H. annuus*, primers were designed in conserved regions of genes.

A total of 18 primer pairs was designed by Primer3 software, that collectively amplified the total chloroplast genome of *J. vulgaris* with overlapping fragments resulting in amplicons between 5808 and 11 110 base pairs (see supplementary Table 1 for primer sequences). For amplification, the Takara La Taq kit (Takara bio inc., Otsu, Shiga, Japan) was used. PCR was carried out in a total volume of 20 µL containing 8-80 ng DNA, 2.5 mM MgCl₂, 2.5 mM of each dNTP, 0.7 µM of each primer and 1 unit Taq DNA polymerase. The PCR cycling conditions were as follows: 1 min at 94°C; 30 cycles of 10 s at 98 °C and 12 min at 69 °C; followed by 10 min at 72 °C. PCR products were loaded on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light to check for amplification. If the PCR products contained more than 1 band, the total product was always loaded on a 1% agarose gel and bands of the right size were cut out of the gel. To extract and purify the DNA fragments from the gel, the Wizard SV gel and PCR Clean-Up System of Promega was used. All cleaned PCR products were run on a gel to estimate the amount of product and in addition the amount of DNA was quantified with an ND-1000 spectrophotometer (Nanodrop Technologies). All 18 amplicons for each individual sample were pooled in equal molar ratios containing roughly 200- 300 nanogram of DNA resulting in 16 pooled samples of 75 µL each.

Sequencing

For sequencing of the cpDNA, three lanes on an Illumina sequencer (Illumina 1G/Solexa, Illumina Inc., San Diego, CA) were used. Sequencing was carried out at the Leiden Genome Technology Center. In the first lane, the DNA isolated from the chloroplasts of sample 17 was run with paired-end reads of 32 basepairs. In the second lane the pooled long range PCR products of samples 1 to 13, 15 and 16 were run and in the third lane sample 14. Both were single-end runs of 35 basepairs (Table 1). Sample 14 was run in a separate lane because of its low DNA concentration. Preparation of all products was done following the protocol of Illumina kits with minor modifications. For sample 17, DNA was fragmented by a nebulizer using 32 psi N₂ for 6 minutes. After purification, the DNA was eluted in 15µL elution buffer. The samples were blunt-ended with T4 DNA polymerase, Klenow polymerase and T4 polynucleotide kinase. After purification, an A-residue was added to the 3-end of the DNA fragments using Klenow fragment (3' to 5' exo minus). Purification was done with a Qiagen MinElute column. Adapters of the paired-end adapter oligo mix were ligated to the DNA fragments. After purification with a Qiagen MinElute column, adapter-ligated DNAs in the range of 200-250bp were size selected using agarose electrophoresis. Products were isolated from the gel using a QIAquick Gel Extraction Kit and after purification a PCR was done.

For samples 1 to 16 (Table 1), sonication with a bioruptor was used to fragment the DNA. This machine was placed in a room at 4°C and was kept cool by adding ice. For a total of 15 minutes, the machine was set on 30 seconds active and 30 seconds inactive. This sonication step was repeated four times. All other steps were the same as done for sample 17 except for the PCR step. Unique index tags of six bases provided in the Multiplexing Sample Preparation Oligonucleotide kit were added in the PCR step to discriminate between the 16 samples. The amplified libraries were quantified by lab-on-a-chip (Agilent Technologies) followed by equimolar mixing of 10 nM per sample. Cluster generation was performed after applying 6 pM of each sample to the individual lanes of the Illumina flow cell and sequencing was carried out on the Illumina Genome Analyzer according to the manufacturer's instructions. Image analysis and base-calling were performed using the Illumina Pipeline 1.3.2, where sequence tags were obtained after purity filtering. This was followed by an alignment using MAQ.

Data filtering and genome assembly

Sample 17 from the first lane was used to assemble a draft cp genome of *J. vulgaris*. The software package MAQ v0.5.0 was used to map all quality-filtered paired reads of the first run against the chloroplast genome of *H. annuus*. To solve gaps in this consensus sequence, a *de novo* assembly was done with the same data using the software package Velvet v 0.6 (parameters: hashlength = 21), which produced 37 747 contigs. To find contigs with homology to the reference, these contigs were aligned to the *H. annuus* reference sequence with the program Mummer v3.0. The contigs having homology to the reference were extended by using the original reads with Velvet. These extended contigs were aligned to the reference of *H. annuus* with Mummer once again, and the contigs which assembled properly were saved. These final contigs were aligned against the consensus sequence; as a result some of the gaps in the consensus were solved. A new MAQ alignment was performed, mapping all the Illumina reads against the last consensus sequence made, to produce the draft sequence.

Bridging the gaps that were still in the draft sequence

The draft sequence still contained 23 gaps with an average gap length of 394 base pairs. Gaps were bridged by adding the data from the runs of the cpDNA amplified by long range PCR of 16 individuals. These data were used in Velvet to produce a *de novo* sequence (parameters: hashlength = 21, short fastq reads). The resulting *de novo* contigs were aligned against the draft sequence in Blast's bl2seq multiple sequence aligner. In this way, five gaps with a total of 1822 basepairs were bridged. The last 18 gaps were bridged by developing primers around the gaps, and traditional Sanger sequencing to yield the final complete cp genome.

Annotation

The program DOGMA was used for annotating all genes and to identify rRNAs and tRNAs. A circular cp genome map (Fig. 1) was drawn using the program GenomeVx.

Comparison of the chloroplast DNA of *J. vulgaris* with other Asteraceae genomes analysed

A total of 22 conserved protein-coding genes from five species, extracted from all available complete chloroplast genomes from Asteraceae deposited at NCBI GenBank (*Helianthus annuus*, NC007977; *Lactuca Sativa*, DQ383816; *Parthenium argentatum*, GU120098; *Guizotia abyssinica*, EU549769 and *Jacobaea vulgaris*, HQ234669), were aligned using the pairwise automatic alignment tool in MacClade 4.06 with further adjustment by hand. To get insight in the informative character of the selected protein-coding genes Maximum Parsimony analyses were run on the individual alignments comprising a total of 33 669 basepairs (bp) with PAUP* 4.0b10 using heuristic search, random addition with 100 replicates, and TBR swapping. The relative robustness for clades found in all single Most Parsimonious Trees (MPTs) was assessed by performing 1000 replicates of bootstrapping using fast, stepwise additions, TBR branch-swapping with 10 random taxon additions per replicate, MULTREES on, and holding 100 trees per replicate. We also calculated tree lengths and CI and RI values measuring the extent of homoplasy.

Detection of polymorphic loci

For visualizing the output of all reads, Mapview was used. This program visualizes all reads that are mapped against the reference genome. Furthermore it can produce a SNP list. The final assembled cp genome was used as a reference. To find SNPs, genomes of individuals 1-17 were used. SNPs were

only added to the list if at least one individual that varied from the reference genome had a coverage of at least 30 reads traversing that particular nucleotide and only when SNPs were located outside A and T polymer regions. Potential microsatellite regions were tracked by looking for 10 or more repeats of A and T nucleotides.

Results and discussion

Construction of the chloroplast genome of *J. vulgaris*

The chloroplast genome of *J. vulgaris* is 150 686 bp in length. The genome contains two inverted repeat (IR) regions of 24 777 bp each. The inverted repeats are separated by a large and small single-copy region (LSC and SSC) of 82 855 and 18 277 bp, respectively. The genome comprises 81 protein-coding genes of which 7 are located in the IRs. *Ycf1* lies partly in the inverted repeat and the single copy region. The four rRNA genes are all located in the IR. There are 29 unique tRNA genes. Twenty two tRNA genes are located in the single copy region whereas the others are located in the IR (Fig. 1).

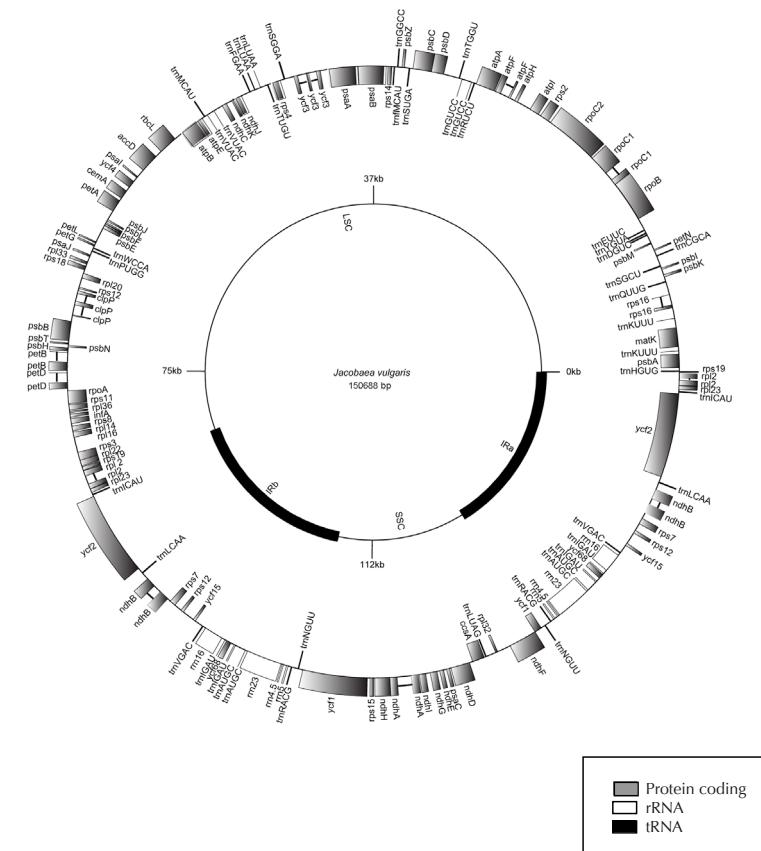


Fig. 1 Representative map of the chloroplast genome of *Jacobaea vulgaris* (Genbank Accession HQ234669).

The single lane on Illumina yielded sufficient reads to map more than 99.9 % of the complete cp genome of *J. vulgaris*. For the pooled individuals, on average 92% of the whole genome was mapped. There was a highly significant correlation between the number of reads and percentage of the genome mapped (Fig. 2). From the figure it is estimated that approximately 1 300 000 single-end Illumina reads of 32 basepair are needed to reach a mapping percentage higher than 99.9% of the cp genome of *J. vulgaris*.

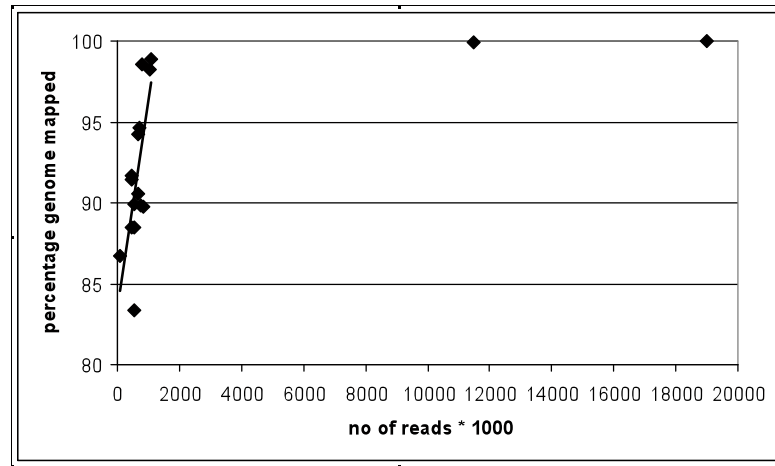


Fig. 2 Number of Illumina sequencing reads plotted against percentage of the chloroplast genome mapped for 17 individuals of *J. vulgaris*. There is a positive relationship between percentage genome mapped and nr of reads if the two points with complete mapping are excluded ($r=71$, $n=15$, $p<0.001$). $y = 0,0126x + 83,544$

Comparison of the sequencing success of cpDNA extracted from chloroplasts with amplified cpDNA using long range PCR

For the first lane with cpDNA extracted from isolated chloroplasts, a paired-end run was carried out on the Illumina platform, yielding 582 Mb of sequence with a read length of 32 bp. Of all reads, only 2.1% (391 604 reads) mapped against the chloroplast genome of *H. annuus*. The obtained reads covered 99.9% of the cp genome of *J. vulgaris* (Table 1, Fig.3). The average coverage was 83 with a coefficient of variation of 0.34 (Fig. 4).

For the other two lanes, containing long range PCR products of 15 individuals in one lane and the long range PCR products of one individual in a separate lane, a single-end run was carried out on the Illumina platform. This run yielded reads of 35 basepairs resulting in 339 and 388 Mb of sequence, respectively. For both lanes more than 99.9% of the reads (96 894 177 and 11 075 400 resp.) mapped against the chloroplast genome of *H. annuus*. In both lanes the reads obtained covered more than 99.9% of the cp genome of *J. vulgaris* (Table 1, Fig. 3). The average coverage obtained for both lanes combined was 4920 x. Average coverage varied largely between primer pairs, with average coverage ranging from 542 x for the lowest to 19755 x for the highest primer pair (Fig.4). The average coefficient of variation of coverage within primer pairs, averaged over all primer pairs, is 1.04 (Fig. 4). In summary, the variation was three times higher than that obtained with the direct cpDNA extraction.

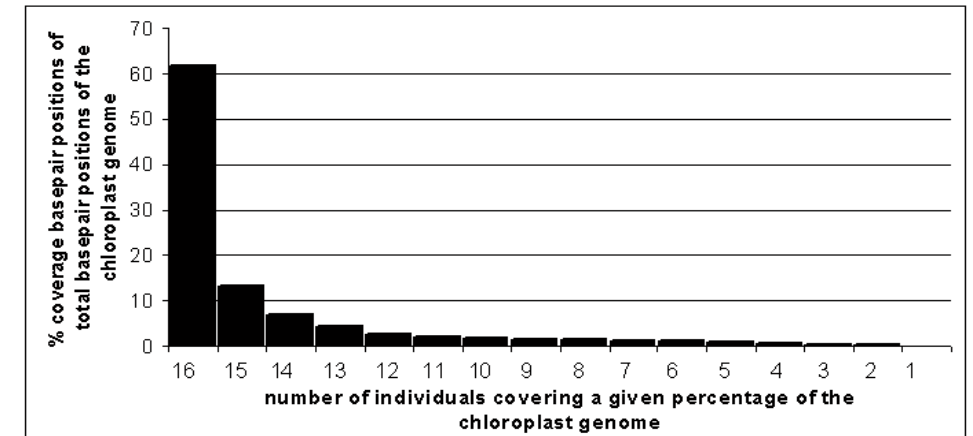


Fig. 3 The column labelled with 1 indicates the number of base pair positions in percentages of total basepair positions that was only covered once in any of these 16 individuals. So, 17 basepairs in the genome were covered by only one individual (less than 0.02 %). To the other extreme the column labelled with 16 indicates the number of base pair positions in percentages of total basepair positions that was covered in all 16 individuals. So, 92804 basepairs were covered by all individuals (more than 60 %).

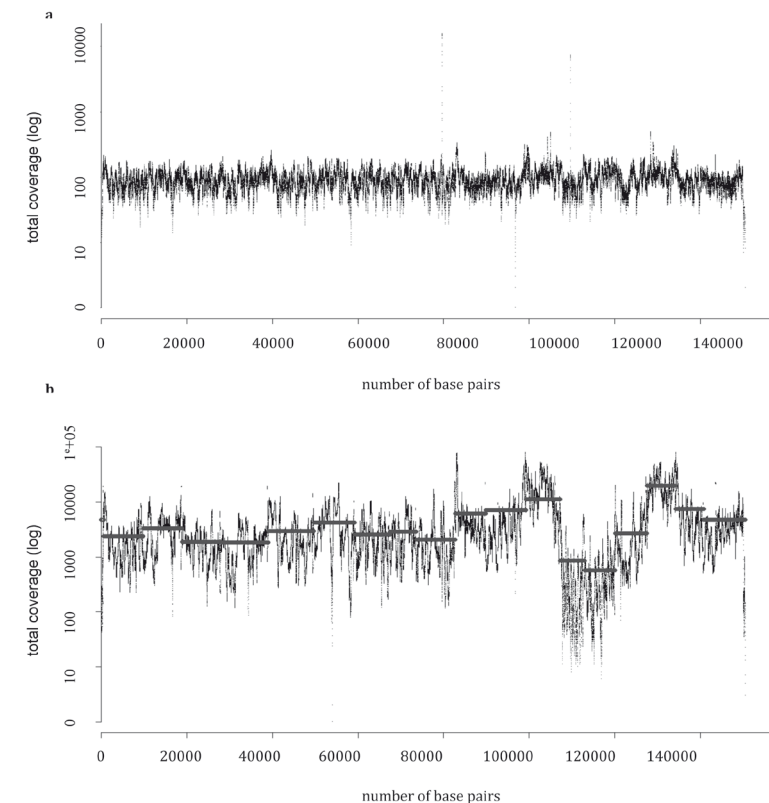


Fig. 4A Whole chloroplast genome coverage plotted for individual 17 of *Jacobaea vulgaris*, of which DNA was obtained by using the chloroplast extraction method. Average coverage = 83 bases per bp and coefficient of variation= 0.34
Fig. 4B Whole chloroplast genome coverage plotted for 16 individuals of *Jacobaea vulgaris* run in two lanes total of which DNA was obtained by using the long range PCR method. The 19 lines plotted indicate the average coverage of every primer. Average coverage= 4920 bases per bp and coefficient of variation= 1.04.

However, extraction of chloroplasts and subsequent extraction of DNA from these chloroplasts was not very efficient for sequencing the complete chloroplast genome. The cpDNA extract still contained around 98% of non cpDNA. The low efficiency of the chloroplast extraction method might be due to the fact that (nuclear) DNA sticks to the surface of the chloroplast or to a shortage of DNAses to remove DNA in the intact chloroplast solution. Furthermore the low efficiency can be caused by poor lysis of the chloroplasts. In contrast, the cpDNA amplified with long range PCR contained less than 1% non cpDNA. Apparently, long range PCR worked very efficiently in *J. vulgaris* and results were much better than the results obtained with the same method for *Pinus* cpDNA sequencing where non cpDNA ranged from 19 to 24%.

Although the number of cpDNA reads obtained with the chloroplast extraction method was far lower than that obtained with long range PCR methods, the variation in coverage over the total chloroplast genome was approximately 3 times lower (Fig 4). Moreover, the variation in coverage of the long range PCR products was primer dependent (see Fig. 4B). Despite the higher variation in coverage, using long range PCR products as templates for Illumina sequencing was far more efficient than using cpDNA directly. Moreover, the cpDNA extraction method proved to be cumbersome because we needed 30 grams of fresh material per individual.

Comparison of the chloroplast DNA of *J. vulgaris* with other Asteraceae genomes analysed

When comparing the full chloroplast genome of *J. vulgaris* with all complete Asteraceae chloroplast genomes (including those from *Guizotia abyssinica*, *Helianthus annuus*, *Lactuca sativa* and *Parthenium argentatum*), a few regions (*trnS-trnC* and *trnE-rpoB*) could not be aligned because these regions were absent in *Parthenium argentatum*, and most other regions showed almost no sequence divergence. Regions that could be aligned and that showed moderate sequence divergence between these five species are listed in Table 4. Five markers (*ndhC-trnV*, *ndhC-atpE*, *rps18-rpl20*, *clpP* and *psbM-trnD*) contained parsimony-informative characters higher than two percent and contained equally high phylogenetic information when compared with other phylogenetic markers that are frequently applied among Asteraceae species such as *trnL-trnF* (6.9%), *trnH-psbA* (1.7%), *rbcl* (1.4%), *rps16* (0.5%) and *ndhF* (0.4%). In Figure 5, the corresponding single MPTs are depicted.

In a former comparison with *H. annuus* against *L. sativa* and with *H. annuus* against *G. abyssinica*, the regions *ndhC-trnV* and *clpP* were already identified as divergent regions within the Asteraceae. CI indexes of the newly discovered phylogenetic markers, indicating homoplasy, of the newly discovered markers were all in the same range as the commonly used markers except for *ycf3-trnS* and *cemA*, which had slightly lower values. RI values ranged from 0.52 to 0.83 for the commonly used markers and from 0.50 to 1.00 for the newly discovered markers.

Analysis of all 22 regions combined resulted in a congruent topology with high support for all internal nodes. Gene trees can be incongruent with species trees when evolution of genes and species did not occur congruently. Gene trees of five regions (*trnL-trnF*, *clpP*, *psbM-trnD*, *rps8-rps14* and *rps15*) were found to be incongruent with the generally inferred species tree of the Asteraceae species analysed (Table 4; Fig. 5).

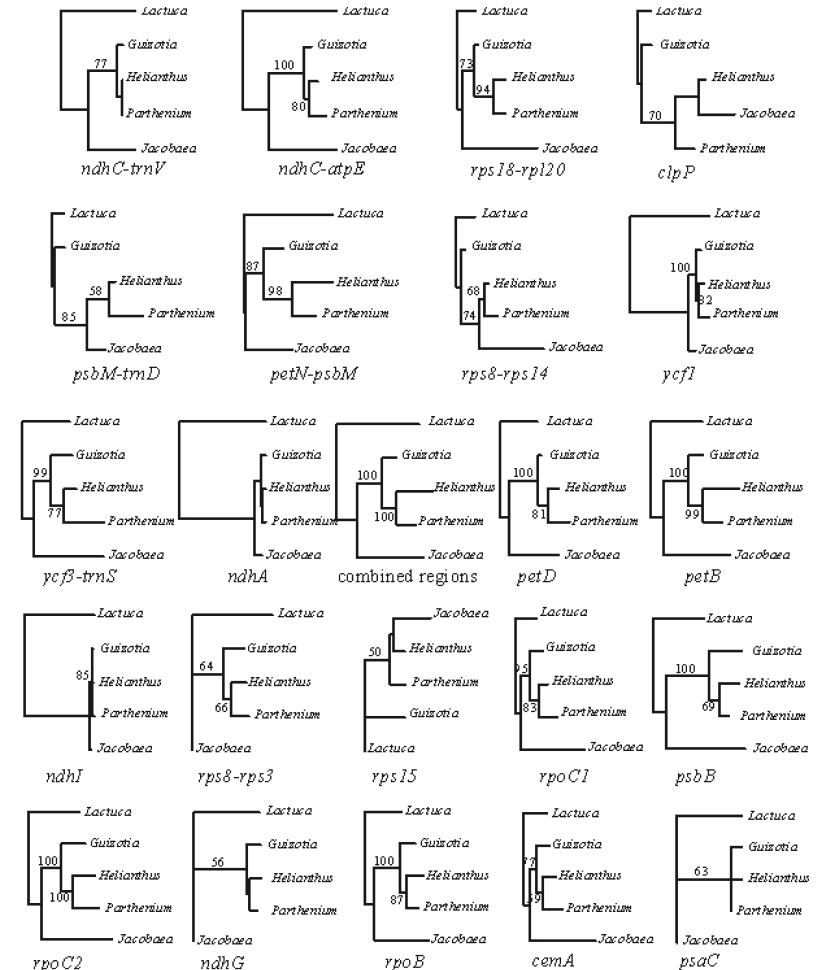


Fig. 5 Phylograms derived from Maximum Parsimony (MP) analysis of alignments of DNA sequences of 5 different Asteraceae species of a total of 27 individual chloroplast regions indicated below the trees. The phylogram called “Combined regions” in the middle is derived from MP analysis of all 27 regions together.

With a length of 150 686 base pairs, *J. vulgaris* has the smallest chloroplast genome compared to the four other Asteraceae cp genomes sequenced so far. The length is 2215 basepairs less than the largest cp genome of *Parthenium argentatum*. The genome is identical in gene content to *Helianthus annuus* and *Lactuca sativa* and differs in gene number with *Guizotia abyssinica* (which has 1 gene less) and *Parthenium argentatum* (which has 4 genes more). Although the similarity in gene content was high, few non-coding regions showed a high sequence divergence between the five Asteraceae species. A number of regions showing sequence divergence between these species contained a high phylogenetic content compared with the standardly applied phylogenetic markers used in the Asteraceae. Those regions seem promising for development of universal primers to further investigate clades in molecular phylogenies of Asteraceae hitherto unresolved. Furthermore, many of these regions are not yet used in angiosperm molecular phylogenetic studies and seem worthwhile to investigate further.

Table 4. Promising regions identified for molecular phylogenetic studies of Asteraceae by comparison of the full chloroplast genomes of *Guizotia abyssinica*, *Helianthus annuus*, *Jacobaea vulgaris*, *Lactuca sativa* and *Parthenium argenatum*.

Region	Length (bp)	Tree length	CI	RI length	Pars. inf. char. (%)	Topologies gene vs. species tree
<i>trnL-trnF</i> ¹	360	100	0.91	0.64	6.9	incongruent
<i>ndhC-trnV</i>	1189	520	0.89	0.88	4	congruent
<i>ndhC-atpE</i>	2376	665	0.96	0.75	3.5	congruent
<i>rps18-rpl20</i>	282	50	0.96	0.78	3	congruent
<i>clpP</i>	889	181	0.97	0.79	2.6	incongruent
<i>psbM-trnD</i>	800	114	0.92	0.55	2.5	incongruent
<i>petN-psbM</i>	569	92	0.97	0.83	2	congruent
<i>rps8-rps14</i>	219	29	0.96	0.75	2	incongruent
<i>ycf1</i>	5811	878	0.94	0.59	2	congruent
<i>ycf3-trnS</i>	1075	232	0.76	0.67	2	congruent
combined regions	40449	7719	0.97	0.62	1.8	congruent
<i>ndhA</i>	2317	208	0.94	0.70	1.7	congruent
<i>trnH-psbA</i> ¹	1571	172	0.92	0.52	1.7	congruent
<i>petD</i>	1266	108	0.97	0.86	1.6	congruent
<i>rbcl</i> ¹	1458	96	0.95	0.76	1.4	congruent
<i>petB</i>	1490	115	0.96	0.75	1.3	congruent
<i>ndhI</i>	547	241	0.95	0.83	1	congruent
<i>rps8-rps3</i>	2451	262	0.94	0.50	1	congruent
<i>rps15</i>	338	27	0.93	0.50	1	incongruent
<i>rpoC1</i>	780	82	0.97	0.80	1	congruent
<i>psbB</i>	1561	78	0.99	0.93	0.8	congruent
<i>rpoC2</i>	4609	260	0.97	0.81	0.8	congruent
<i>ndhG</i>	540	31	1.00	1.00	0.7	congruent
<i>rpoB</i>	3606	133	0.97	0.83	0.6	congruent
<i>rps16</i> ¹	1159	101	0.99	0.83	0.5	congruent
<i>cemA</i>	690	47	0.80	0.75	0.4	congruent
<i>psaC</i>	264	10	1.00	1.00	0.4	congruent
<i>ndhF</i> ¹	2232	156	0.98	0.67	0.4	congruent

The consistency index (CI) and retention index (RI) were calculated with autapomorphic characters excluded.

¹ Commonly used phylogenetic markers included for comparison

Detection of polymorphic loci

Single Nucleotide Polymorphisms (SNPs)

The 17 individuals of *J. vulgaris* yielded a total of 32 SNPs (Table 2A), which is on average 1 SNP per 4705 bp. In 66% of the cases a SNP allele was found only in a single individual. Fifty nine per cent of the SNP polymorphisms where substitutions from a purine to a pyrimidine or vice versa. No SNPs were found in tRNAs (Table 3). Within the single copy region (LSC and SSC) SNPs were almost equally divided over coding DNA (tRNA + exons+ genic) (13) and intergenic spacers and introns (19). However, in the coding DNA, on average 1 SNP every 4573 bp was found compared to 1 SNP on average for every 2780 bp in intergenic and introns spacers (Table 3). Within the genes 2 SNPs were located in introns, this is on average 1 SNP per 3439 bp compared to 1 SNP per 4811 bp located in coding gene sequences (genes + exons) (Table 3). Of the 13 SNPs found in coding DNA, 3 resulted in non-synonymous substitutions (Table 2A).

Table 2. List of positions and variants of single nucleotide polymorphisms and microsatellites in the chloroplast genome of *Jacobaea vulgaris*.

A) SNP positions, alleles with the most occurring allele first, frequency of the least occurring allele in 17 individuals of the cp genome of *J. vulgaris* and region and locus of these SNPs.

Position	Alleles	Freq.	Region	Locus	Position	Alleles	Freq.	Region	Locus
165	T/A	0.13	Intergenic	<i>trnH-GUG/ psbA</i>	61 436	C/T	0.31	Genic	<i>petA</i>
4032	A/C	0.06	Intergenic	<i>matK/ trnK-UUU</i>	65 579	G/C	0.06	Intergenic	<i>trnP-UGG/ psal</i>
5555	A/T	0.13	Intron	<i>rps16</i>	66 056	T/G	0.19	Intergenic	<i>psal/ rpl33</i>
7837	A/C	0.06	Intergenic	<i>psbK/ psbL</i>	67 055	G/A	0.25	Intergenic	<i>Rps18/ rpl20</i>
11 353	C/A	0.06	Intergenic	<i>trnY-GUA/ trnE-UUC</i>	67 963	T/C	0.69	Intergenic	<i>Rpl20/ rps12</i>
18 287	A/C	0.13	Exon	<i>rpoC1</i>	69 567**	T/C	0.06	Exon	<i>clpP</i>
22 648	C/T	0.06	Genic	<i>rpoC2</i>	70 234	T/G	0.06	Intron	<i>clpP</i>
24 906	T/G	0.38	Intergenic	<i>atpI- atpH</i>	92 417*	C/T	0.06	Intergenic	<i>trnL-CAA/ ndhB</i>
31 299	C/A	0.06	Intergenic	<i>trnT-GGU/ psbD</i>	97 496*	C/A	0.06	Intergenic	<i>Rps7/ ycf15</i>
39 790	A/G	0.44	Genic	<i>psaA</i>	106 663*	T/G	0.06	Intergenic	<i>trnR-ACG/ trnN-GUU</i>
39 829	G/A	0.13	Genic	<i>psaA</i>	106 664*	C/A	0.06	Intergenic	<i>trnR-ACG/ trnN-GUU</i>
43 765	C/T	0.06	Intergenic	<i>Ycf3/ trnS- GCA</i>	108 200**	G/C	0.25	Genic	<i>Ycf1</i>
47 181	G/C	0.06	Intergenic	<i>trnL-UAA/ trnF-GAA</i>	118 779	C/G	0.06	Genic	<i>ndhD</i>
49 751	C/T	0.06	Genic	<i>ndhC</i>	123 423	A/C	0.06	Intergenic	<i>Rpl32/ ndhF</i>
53 025	G/A	0.06	Genic	<i>atpB</i>	124 027	C/T	0.06	Genic	<i>ndhF</i>
60 245	C/T	0.06	Genic	<i>cemA</i>	124 035**	C/T	0.06	Genic	<i>ndhF</i>

SNPs that were tested for multiple individuals with high resolution melting are indicated by bold typeface. SNPs located in the Inverted Repeat are indicated with *. Non-synonymous substitutions are indicated with **.

B) Potential microsatellite loci, repeat, repeat length in the consensus chloroplast sequence and the region and locus of these repeats in the cp genome of *J. vulgaris*.

position of repeat	Repeat	Repeat length of consensus	Region	Locus
6705	A	11	Intergenic	<i>rps16/ trnQ- UUG</i>
12459	T	14	Intergenic	<i>trnE-UUC/ rpoB</i>
13143	A	10	Genic	<i>rpoB</i>
16413	T	10	Intron	<i>rpoC1</i>
17759	A	10	Exon	<i>rpoC1</i>
18185	A	10	Exon	<i>rpoC1</i>
24848	A	17	Intergenic	<i>atpL/ atpH</i>
27760	T	15	Intergenic	<i>atpF/ atpA</i>
27776	A	11	Intergenic	<i>atpF/ atpA</i>
34901	A	10	Intergenic	<i>trnS- UGA/ psbZ</i>
41459	T	10	Intergenic	<i>psaA- ycf3</i>
41471	A	13	Intergenic	<i>psaA- ycf3</i>
46228	A	14	Intergenic	<i>trnT-UGU/ trnL-UAA</i>
49996	G	11	Intergenic	<i>ndhC/ trnV-UAC</i>
53630	A	10	Intergenic	<i>atpB/ rbcl</i>
54013	T	18	Intergenic	<i>atpB/ rbcl</i>
58662	T	10	Intergenic	<i>psaL/ ycf4</i>
64247	A	11	Intergenic	<i>psbIE/ petL</i>
69969	A	11	Intron	<i>clpP</i>
70312	A	10	Intron	<i>clpP</i>
72916	A	11	Genic/Intergenic	<i>psbT/ psbN</i>
74047	A	11	Intron	<i>petB</i>
76775	T	17	Genic	<i>rpoA</i>
79191	T	13	Intergenic	<i>rps8/ rpl14</i>
79774	A	10	Intergenic	<i>rpl14/ rpl16</i>
81396	T	10	Intergenic	<i>rpl16/ rps3</i>
82909*	T	10	Intergenic	<i>rps19/ rpl2</i>
109743	A	10	Genic	<i>ycf1</i>
112000	A	11	Genic	<i>ycf1</i>
114539	T	10	Intron	<i>ndhA</i>
121458	A	11	Intergenic	<i>ccsA/ trnL- UAG</i>
121889	T	11	Intergenic	<i>trnL- UAG/ rpl32</i>
123661	A	10	Intergenic	<i>rpl32/ ndhF</i>
150626*	A	10	Intergenic	<i>rpl2/ trnH-GUG</i>

Microsatellites that were tested for polymorphisms in multiple individuals are indicated by bold typeface. Microsatellites located in the Inverted Repeat are indicated with *.

Table 3. Summary of number of basepairs, number of SNPs, number of basepairs per SNP, number of microsatellite regions and number of basepairs per microsatellite region.

	nr of bp		nr of SNPs		nr of bp/nr of SNPs		nr of ms		nr of bp/nr of ms	
	SCR	IR's	SCR	IR's	SCR	IR	SCR	IR's	SCR	IR
non coding DNA	41 688	8574	16	4	2606	2144	28	1	1489	8574
coding DNA	59 445	11 688	12	0	4954		5	0	11 889	
rRNA	0	4515	0	0			0	0		
non coding gene	6877	1339	2	0	3439		5	0	1375	
coding gene	57 733	11 174	12	0	4811		6	0	9622	

Reads derived from the inverted repeats are distributed randomly to IRa or IRb by the assembly software. However if IRa is different from IRb by an indel or SNP this would be observed as a polymorphism within an individual. That was however never observed as we specifically checked for this. In the one case where we found that the sequence of the inverted repeat of individual 11 was deviating from other individuals for 4 positions, these positions within individual 11 were fully homozygous in both IRa and IRb. All SNPs found in the Inverted Repeats, 2 x 4 in total, were located in the intergenic spacers of individual 11. The 4 SNPs found in individual 11 in IRa were found in exactly the same place and the same mutation as in IRb. This suggests “concerted evolution” or gene conversion for the inverted repeat region. On average 1 SNP every 1808 bp was found in the intergenic spacers in the IR. For a subset of 11 SNPs, primers were developed (Table 2A) and several individuals were genotyped using high resolution melting. For all these individuals, the SNP polymorphisms were confirmed.

The number of SNPs that were found in this study might be slightly underestimated because the whole cp genome was not mapped with sufficient coverage to detect all SNPs in the 17 individuals analysed. Although the number of synonymous substitutions in chloroplast genes is on average at least three times lower than that of nuclear genes²⁴, we still found SNPs using chloroplast genomes of 17 individuals of *J. vulgaris* originating from different populations. We found that SNPs were 1.8 times more frequent in intergenic spacers and introns as compared to DNA coding genes. These findings are in line with the assumption that coding DNA generally evolves more slowly than non-coding regions.

The result that individual 11 has 4 SNPs in both inverted repeat regions suggest that a mechanism is present that provides simultaneous mutations in both IRa and IRb. In all 17 individuals the sequences of IRa and IRb did not differ from each other by a single base. The gene *Ycf1* starts at the end of IRb and extends into SSR, to yield the full *Ycf1* sequence. In IRa the *Ycf1* gene starts but is not extended into SSR yielding a non-functional sequence. It suggests that there is a selective force that prevents that the inverted repeat regions start to deviate from each other even when all the mutations are located in the intergenic spacer or non-functional genes. As a consequence the inverted repeats may contribute to the structural stability of the cp genome. Two plant groups, legumes and conifers, lost their inverted repeat and comparative sequence studies showed that these chloroplasts experienced a four fold increase in silent substitutions compared with chloroplasts containing the inverted repeat.

Microsatellites

A total of 34 microsatellite regions were found with A/T repeats longer than 9 repeats, which is 1 microsatellite per 4432 basepairs. Only one microsatellite region was found with 11 G repeats and no repeats of 10 or more Cs were found in the chloroplast genome of *J. vulgaris*.

Within the single copy region 5.6 times as much microsatellite regions were found in intergenic spacers and introns compared to coding DNA (28 against 5 respectively). No microsatellites were found in the tRNA and rRNA. We found on average one microsatellite region every 1489 bp in intergenic spacers and introns against 1 out of 11 889 bp in coding DNA (Table 3). Within the genes microsatellite regions were almost equally divided over exons and genes (6) and introns (5). This is on average 1 out of 1375 bp for introns against 1 out of 96 222 bp for exons and genes (Table 3). This is not in accordance with the data of SNPs where the number of SNPs per bp was relatively almost the same for exons+ genes and introns. An insertion or a deletion in an exon or gene will lead to a frame shift and therefore likely leads to a non-functional protein. Both microsatellite regions and SNPs occur less in DNA coding regions (exons +genes +tRNA) compared to non-coding regions (intergenic spacers + intron). However, this difference is more marked for microsatellite regions than SNPs.

Of the 34 microsatellite regions, only one was located on IRb in an intergenic spacer. This is surprising because concerted evolution, as earlier suggested, should lead to exact sequence duplication in IRa compared to IRb, and therefore both Inverted Repeats should contain the same number of nucleotide repeats. Indeed we found a microsatellite region at the same place on both IRs, but this repeat was only 8 basepairs on IRa and is therefore not included in Table 2B. For 10 repeat regions, primers were developed and multiple individuals from different populations were genotyped (Table 2B). Optimization failed for one primer pair, but the other 9 regions were amplified and they were all polymorphic. We tested 93 *J. vulgaris* individuals in total and found that all were polymorphic with the number of alleles per locus varying from two to six with an average of 3.3 alleles per locus.

The number of microsatellite regions is promising for investigating allele frequencies in populations and eventually, together with the SNP data, tracing the source population(s) of non-native *J. vulgaris*. The number of variable microsatellites might be higher since we arbitrarily decided to include only mononucleotide repeats that were at least 10 base pairs long. We found that potential microsatellite regions were 4.7 times more located in intergenic regions and introns compared to coding regions. Because SNPs were only 1.8 times more located in intergenic regions and introns compared to coding regions, we conclude that, point mutations are more frequent in coding DNA than indels leading to frame shifts immediately. Although the location of potential microsatellite loci is certain, the repeat length is an approximation. During long range PCR and PCR steps in the sample preparation steps for the Illumina platform indels can occur in microsatellite loci, leading to less or more repeats. Consequently the Illumina reads for microsatellite loci differed, making it hard to deduce the repeat length. This could also be the explanation for finding a difference in repeat length of a potential microsatellite locus between the IRs.

In conclusion, we found promising regions for development of universal primers that can be used for further investigation of clades in molecular phylogenies of Asteraceae. Considering the number of SNPs and microsatellites found in this study, we recommend screening of the complete chloroplast genome to

find differences within a species. Despite the higher variation in coverage, using long range PCR products as templates for Illumina sequencing, seemed to be far more efficient than using cpDNA directly.

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Supporting Information

Table S1

Primers used for amplifying the chloroplast genome of *Jacobaea vulgaris* with long range PCR.

Primer name	Start position (bp) in cp genome <i>J. vulgaris</i>	Primer sequence (5'>3')	Tm in C
1F	400	GAG CAT TAC GTT CAT GCA TAA CTT CCA TAC CAA GG	72.8
1R	9794	TCC ACT TCT TCC CCA TAC TAC GAG TGA AAG AGA AA	72.1
2 F	9287	GAC TGC AAA TCC TTT TTC CCC AGT TCA AAT C	71.6
2 R	18772	CGG TAG CTT GTC GGA AAC CCA GAG TCT TTA CT	72.2
3 F	18683	AAA GTG ATA GAC GGA ACT GCC ATG AAA CGA CT	71.9
3 R	27901	TGA ATA CGA GCA ATG CCG TCA CCT ACT TGA	73.3
4 F	27822	CCG ACG AAA TTA GTA ATA TTA TCC GCG AAC GTA	70.1
4 R	38959	TTT TCC TAG GTG CTC ATT TTG TAT GGG CTT TT	70.4
5 F	38857	CGG CTG GGT AGC AGG AGC AAC TTT TAA TTT AT	70.14
5 R	49604	ACG GTT TTT CTT TAT CCA TGG GCA ATG AGT TT	71.7
6 F	49517	CCC TTT CGC CAT GCA TAA ACT AAA CCA ACA AT	72.5
6 R	59127	CCA AGA TAA CTC GAG GTT CCA ACC AAC AAG AA	72.0
7 F	59072	TCG AAA AAC AGG TAA TTT CTG CTG GGC TGT TA	72.0
7 R	67422	AAT CAA TTT TCG TCG TTT GTG GAT TAC TCG AA	70.5
8 F	67238	GCG GTG GAT TCC TTT CAA CTT ACT TCT TTT ATG A	70.7
8 R	73610	CCA TCT CTA TCA AGA TGA CAG ACC CAT TCT CTG	70.7
9 F	73524	TTG GTA GTT CGA CCG TGG AAT TTC TTT GTT TC	71.7
9 R	82677	GGA TCG TAT GGT AGG ACA CAA ATT GGG AGA AT	71.0
10 F	82624	GCG AGA TCT ATT ATC GCT TTT TGC ATG TCC TC	71.6
10 R	89850	CCT TGA GAC TTG TTA TCC AGG AAC TTG TTC AGA	70.2
11 F	89724	CTT TTC GCT CCG CTT AGC CTT ATC CCT CTC TA	72.6
11 R	98855	CTC CCA AGG GCA GGT TCT TAC GCG TTA CTC	74.1
12 F	98754	GAA TCT CAT GGA GAG TTC GAT CCT GGC TCA	73.3
12 R	107188	TCC ATA ACG TGA GCT CGG AGA AGG AAG AGA TA	72.1
13 F	107123	TCA ATT CGG TCG TTG TGG TCG GAC TCT ATT AT	72.2
13 R	112952	TGC TCT GGC CCA TTT ACA GTT ATT GCT TCT GT	72.4
14 F	112751	ATG ATA GTC AAT ATG GGA CCT CAC CAC CCA TC	71.9
14 R	120095	ATA GAA ACA AAT AAC TCG CGC GGT CCA GAA TC	72.4
15 F	120036	TGT CAC GGC AGA TGT TCT ATG GAT ACA AGT TAT TT	70.31
15 R	127553	ACC CCA ATT ATG ACA TCC CTT CTC TCC CAC TT	73.0
16 F	127382	TTC GTA GCC ACG TGC TCT AAT CCT CTG AGC TA	72.8
16 R	134278	TAA TAC AGA GGA TGC AAG CGT TAT CCG GAA TG	72.3
17 F	134174	CCC TAC CGT ACT CCA GCT TGG TAG TTT CCA C	71.8
17 R	140762	GGG CGG AAC AGA TCT ACT AAT TCT TTG ATT CCA	71.7
18 F	140596	AAG ATC CCC TTT AAG ATC AAA CAA TTC CAT CG	70.2
18 R	433	CAC TTG GGC TGA TAT CAT TAA CCG TGC TAA CC	71.9