High-Throughput Discovery of Chloroplast and Mitochondrial DNA Polymorphisms in Brassicaceae Species by ORG-EcoTILLING

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Abstract

Background: Information on polymorphic DNA in organelle genomes is essential for evolutionary and ecological studies. However, it is challenging to perform high-throughput investigations of chloroplast and mitochondrial DNA polymorphisms. In recent years, EcoTILLING stands out as one of the most universal, low-cost, and high-throughput reverse genetic methods, and the identification of natural genetic variants can provide much information about gene function, association mapping and linkage disequilibrium analysis and species evolution. Until now, no report exists on whether this method is applicable to organelle genomes and to what extent it can be used.

Methodology/Principal Findings: To address this problem, we adapted the CEL I-based heteroduplex cleavage strategy used in Targeting Induced Local Lesions in Genomes (TILLING) for the discovery of nucleotide polymorphisms in organelle genomes. To assess the applicability and accuracy of this technology, designated ORG-EcoTILLING, at different taxonomic levels, we sampled two sets of taxa representing accessions from the Brassicaceae with three chloroplast genes (accD, matK and rbcL) and one mitochondrial gene (atp6). The method successfully detected nine, six and one mutation sites in the accD, matK and rbcL genes, respectively, in 96 Brassica accessions. These mutations were confirmed by DNA sequencing, with 100% accuracy at both inter- and intraspecific levels. We also detected 44 putative mutations in accD in 91 accessions from 45 species and 29 genera of seven tribes. Compared with DNA sequencing results, the false negative rate was 36%. However, 17 SNPs detected in atp6 were completely identical to the sequencing results.

Conclusions/Significance: These results suggest that ORG-EcoTILLING is a powerful and cost-effective alternative method for high-throughput genome-wide assessment of inter- and intraspecific chloroplast and mitochondrial DNA polymorphisms. It will play an important role in evolutionary and ecological biology studies, in identification of related genes associated with agronomic importance such as high yield and improved cytoplasmic quality, and for identifying mitochondrial point mutations responsible for diseases in humans and other animals.

Introduction

The fields of ecology and phylogeny are currently experiencing a renaissance spurred by the rapid development of molecular detection techniques. By detecting genetic variation in both nuclear and organelle genomes, molecular markers have made a profound and significant contribution to studies of evolution, domestication, speciation, evolution of genomes, genetic diversity, population structure, levels of gene flow, patterns of historical biogeography and analyses of parental assignments, genetic variability and inbreeding [1–6]. Mitochondria and chloroplasts are important organelles in eukaryotic organisms, and both genomes contain two vital sets of genes [7]. Plant mitochondrial (mt) DNAs are extremely variable in size (200–2400 kb) [8,9], whereas animal mtDNAs are essentially invariant in gene order among all vertebrates [10]. Chloroplasts (cp) contain their own small genomes, which averages 120 to 200 kilobase pairs (kb) among almost all chloroplast-containing organisms [11]. The plant chloroplast genome shares many features with animal mtDNA and the two have been referred to as ‘natural counterparts’ [12]. In animals, mtDNA is characterized by a small size, high copy number, relatively conserved gene order, ready availability of primers and rapid substitution rates [13], whereas in plants, the chloroplast genome is associated with a conserved gene order, widespread availability of primers and a general lack of heteroplasmy and recombination [14]. As a result,
the nonrecombinant, uniparentally inherited and effectively haploid nature of chloroplast and mitochondrial genomes makes them useful tools for studies on plant and animal evolution, respectively [14]. Chloroplast genomes are predominantly maternally inherited (mainly transmitted through the embryos of seeds) and so can reveal maternal lineages [15–18], enabling divergent patterns of variation to be detected in these genomes compared with those revealed by nuclear markers. For example, investigation of chloroplast genomes can be used to document the maternal parent of hybrid plants [19], define organelle haplotypes [20] or detect introgression [21,22].

In order to detect informative polymorphisms for phylogenetic studies, a large number of molecular techniques have been developed for assessing chloroplast and mitochondrial genomes. Conventional methods include DNA restriction mapping [23,24], the use of hybridization-based restriction fragment length polymorphisms (RFLP) [25], PCR-RFLP [26–28] and nucleotide sequence analysis [29,30]. However, each of these methods have some disadvantages, including low resolution, labor intensity and the requirement for a large amount of isolated DNA. For instance, although Chloroplast microsatellites (cpSSR) have proven to be useful markers for gaining insights into the genetic relationships of closely related species and populations [31,14], they are limited to study of closely related taxa where there are no or few nucleotide substitutions, especially in the coding regions of the chloroplast genome [32].

Because single-base substitutions and small insertions and deletions (INDELs) are the most common forms of genetic variation in organelle genomes, nucleotide sequence analysis of specific chloroplast and mitochondrial genes has been widely used in phylogenetic and ecological studies. Several key genes, such as chloroplast rbcL, matK and accD, mitochondrial atp6 and a mitochondrial fragment COX1 play important roles in clarifying phylogenetic relationships amongst plants [33–37]. However, rbcL, matK and accD only represent about 2.9% of the genome. Next-generation sequencing technologies, such as Roche/454, Illumina/Solexa and Life/APG are able to generate large volumes of sequence data at relatively low cost [38], enabling complete coverage of organelle genomes. However, at present this is only feasible for relatively low level sampling of individuals [39,40]. This has led to a debate over the relative worth of taxon sampling (investigating many lineages) compared with site sampling (investigating many sites from many genes from a few crucial lineages) in building phylogenetic trees from sequence data [41,42]. Thus there is a need for new methods that have the features of high resolution, high throughput, and ease of use, accuracy and cost effectiveness.

TILLING (Targeting Induced Local Lesions in Genomes) and EcoTILLING were developed as a means for high-throughput discovery of DNA polymorphisms in nuclear genomes in EMS-mutagenized [43–47] and natural populations [48]. The initial development of these methods employed the mismatch-specific endonuclease CEL I to discover point mutations in genes, in conjunction with PCR-based screening [49,50]. Subsequently there have been modifications of the approach using other detection methods, including the use of new generation sequencing coupled with multidimensional pooling for the identification of rare alleles in populations of rice and wheat [51].

Given the ability of CEL1 and a PCR-based strategy to detect single nucleotide polymorphisms (SNPs), we decided to explore whether this approach could be applied to organelle genomes. We therefore developed a method, designated “ORG-EcoTILLING”, for high-resolution, high-throughput detection of multiple polymorphisms in chloroplast and mitochondrial genes.

In order to assess the applicability and accuracy of ORG-EcoTILLING we sampled two sets of plant taxa representing different levels of variation within the Brassicaceae. The Brassicaceae include approximately 340 genera and 3350 species [52], of which the model plant Arabidopsis thaliana and domesticated species such as oilseed rape, Chinese cabbage, broccoli, turnip, radish, mustard, and other Brassica crops, are perhaps the most familiar [53]. Plants within the Brassicaceae have therefore been regarded as ideal materials for the study of evolutionary relationships [54,55]. This study aimed to (1) develop a strategy for ORG-EcoTILLING to discriminate genetic SNPs and INDELs in chloroplast and mitochondrial genes of numerous Brassicaceae plant accessions; (2) assess the accuracy of ORG-EcoTILLING for the identification of cytoplasmic DNA Polymorphism; and (3) provide an effective, low-cost, high-throughput technology for identification of genes associated with agronomic importance such as high yield and improved cytoplasmic efficiency, analyzing phylogenetic relationships in plants, and identifying point mutations of mitochondrial genes responsible for diseases in humans and other animals.

Materials and Methods

Plant material

To evaluate the applicability and accuracy of ORG-EcoTILLING for detecting DNA variation in chloroplast genes at different taxonomic levels, two sets of plant specimens were sampled. The first set, composed of 91 representative accessions from 45 species and 29 genera of seven tribes (Trib. Brassiceae Hayek, Trib. Sisymbriaceae DC, Trib. Matthioliaceae O.E. Schulz, Trib. Alyssaceae Gren. Et Godr, Trib. Arabidiceae DC, Trib. Hesperideae Prantl, and Trib. Lepidiceae DC) (Table 1) was used to assess the applicability and accuracy at intertribal, intergeneric and interspecific levels. The second set sampled 90 accessions of cultivars of Brassica napus, and three accessions each of B. rapa and B. oleracea (Table 2), which were used to evaluate the applicability and accuracy of this method at intraspecific and interspecific levels.

ORG-EcoTILLING procedure

The ORG-EcoTILLING procedure includes six basic steps (Figure 1), modified from Haughn and Gilchrist (2006) [56]. Firstly, total DNA is extracted from above 181 accessions from the natural population and is normalized. Query DNA and reference DNA are amplified by allele-specific primers with M13F and M13R adaptors, respectively. Then the PCR products of query DNA and reference DNA are mixed in a 1:1 ratio. The mixture is amplified using a forward primer with 700 nm dye label and a reverse primer with an 800 nm dye label attached to the 5’ ends. Thirdly, the PCR products are heated and cooled to form heteroduplexes between query DNA and reference DNA in the pool. Finally, the PCR products are heated and cooled to form heteroduplexes between query DNA and reference DNA in the pool. The PCR products are heated and cooled to form heteroduplexes between query DNA and reference DNA in the pool. Because the nuclease cleaves either of the two strands arbitrarily, cleavage products can be detected in both the IRD700 and IRD800 channels of the gel image.

DNA extraction

Total DNA was extracted from leaves from seedlings according to the methods described by Murray and Thompson [57]. DNA from each accession of 50 pooled plants was normalized to a final
### Table 1. 91 accessions from Cruciferae used for ORG- EcoTILLING.

| Sample code | Materials name           | Origin     | Trib           | Genus  | Species                  |
|-------------|--------------------------|------------|----------------|--------|--------------------------|
| A1          | Changyouxiaohouloucai    | China      | Trib. Brassiceae Hayek | Brassica | B. rapa                  |
| A2          | Xishui                   | China      | Trib. Brassiceae Hayek | Brassica | B. rapa                  |
| A3          | Guangfuqing              | China      | Trib. Brassiceae Hayek | Brassica | B. rapa                  |
| A4          | Baichengbaiyoucai        | China      | Trib. Brassiceae Hayek | Brassica | B. rapa                  |
| A5          | Ling chuanyoucai         | China      | Trib. Brassiceae Hayek | Brassica | B. rapa                  |
| A6          | Qu xu                    | China      | Trib. Brassiceae Hayek | Brassica | B. rapa                  |
| A7          | Jiningtianjinlin         | China      | Trib. Brassiceae Hayek | Brassica | B. rapa                  |
| A8          | Plaoerbai                | China      | Trib. Brassiceae Hayek | Brassica | B. rapa                  |
| A9          | Niuyezhongshuaixin       | China      | Trib. Brassiceae Hayek | Brassica | B. parachinensis L.H.Bailey |
| A10         | Wutacai                  | China      | Trib. Brassiceae Hayek | Brassica | B. napinoxa               |
| A11         | Taicai                   | China      | Trib. Brassiceae Hayek | Brassica | B. rapa                  |
| A12         | Lelingwuqing             | China      | Trib. Brassiceae Hayek | Brassica | B. rapa                  |
| A13         | Majrova “Petrovski”      | Sweden     | Trib. Brassiceae Hayek | Brassica | B. rapa                  |
| A14         | Majrova “Purple Top Milan” | Sweden   | Trib. Brassiceae Hayek | Brassica | B. rapa                  |
| A15         | Tradkali “Jersey Walking Stick” | Sweden | Trib. Brassiceae Hayek | Brassica | B. oleracea               |
| A16         | Kaliot Lanttu “Wilhelmsburger” | Sweden | Trib. Brassiceae Hayek | Brassica | B. napus                  |
| A17         | Majrova Nauris “Goldhall” | Sweden   | Trib. Brassiceae Hayek | Brassica | B. napus                  |
| A18         | Midasi                   | Canada     | Trib. Brassiceae Hayek | Brassica | B. napus                  |
| A19         | H47                      | Russia Union | Trib. Brassiceae Hayek | Brassica | B. napus                  |
| A20         | Qikuzhen                 | Japan      | Trib. Brassiceae Hayek | Brassica | B. napus                  |
| A21         | Zhongshuan-4             | China      | Trib. Brassiceae Hayek | Brassica | B. napus                  |
| A22         | Zhongshuan-4NSA          | China      | Trib. Brassiceae Hayek | Brassica | B. rapa                  |
| A23         | Baoziganlan              | China      | Trib. Brassiceae Hayek | Brassica | B. oleracea               |
| A24         | Ziglanlan                | China      | Trib. Brassiceae Hayek | Brassica | B. oleracea               |
| A25         | Shimianlianhuabai        | China      | Trib. Brassiceae Hayek | Brassica | B. oleracea               |
| A26         | Baipilin                 | China      | Trib. Brassiceae Hayek | Brassica | B. oleracea               |
| A27         | Tuanyexiaohuaicai        | China      | Trib. Brassiceae Hayek | Brassica | B. oleracea               |
| A28         | Livqinghuacai            | China      | Trib. Brassiceae Hayek | Brassica | B. oleracea               |
| A29         | Zhonghuaijelan           | China      | Trib. Brassiceae Hayek | Brassica | B. oleracea               |
| A30         | Yeshenggianlan           | China      | Trib. Brassiceae Hayek | Brassica | B. oleracea               |
| A31         | Yuyigianlan              | China      | Trib. Brassiceae Hayek | Brassica | B. oleracea               |
| A32         | Nanfangren               | Russia Union | Trib. Brassiceae Hayek | Brassica | B. juncea                 |
| A33         | Bangcai02                | China      | Trib. Brassiceae Hayek | Brassica | B. juncea                 |
| A34         | Banyedatoucai            | China      | Trib. Brassiceae Hayek | Brassica | B. juncea                 |
| A35         | Donghaiaojiaofengweicai  | China      | Trib. Brassiceae Hayek | Brassica | B. juncea                 |
| A36         | Pusa Bold                | India      | Trib. Brassiceae Hayek | Brassica | B. juncea                 |
| A37         | CMS (Mri)                | India      | Trib. Brassiceae Hayek | Brassica | B. juncea                 |
| A38         | Olebra                   | Sweden     | Trib. Brassiceae Hayek | Brassica | B. nigra                  |
| A39         | Hejie                    | Germany    | Trib. Brassiceae Hayek | Sinapis L. | B. nigra                |
| A40         | Xinjiangyeshengyoucai A  | China      | Trib. Brassiceae Hayek | Sinapis L. | S. arvensis               |
| A41         | Xinjiangyeshengyoucai B  | China      | Trib. Brassiceae Hayek | Sinapis L. | S. arvensis               |
| A42         | Bajie                    | China      | Trib. Brassiceae Hayek | Sinapis L. | S. alba                  |
| A43         | Maojiaocai               | China      | Trib. Brassiceae Hayek | Brassica | S. alba                  |
| A44         | Ethiopia jie             | Ethiopia   | Trib. Brassiceae Hayek | Brassica | B. carinata               |
| A45         | Huangziajie              | Ethiopia   | Trib. Brassiceae Hayek | Brassica | B. carinata               |
| A46         | 77-1304                  | USA        | Trib. Brassiceae Hayek | Brassica | B. carinata               |
| A47         | 77-1305                  | USA        | Trib. Brassiceae Hayek | Brassica | B. carinata               |
| A48         | 88-212463                | USA        | Trib. Brassiceae Hayek | Brassica | B. carinata               |
| A49         | 88-203221                | USA        | Trib. Brassiceae Hayek | Brassica | B. carinata               |
| A50         | Yuewenguoloubozi         | China      | Trib. Brassiceae Hayek | Raphanus L. | R. sativus             |
concentration of 30 ng μL⁻¹. Following extraction, DNA samples were arrayed in a 96-well format.

Primer design
Using Primer Premier (PREMIER Biosoft International, http://www.premierbiosoft.com/index.html), two types of primers were designed for PCR amplification: gene-specific primers directly labeled at the 5’ end with IRD 700 and IRD 800 (such as the primer for *atp6*) or primers in which a sequence from the universal primer M13F (CACGACGTTGTAAAACGAC) was added to the 5’ end of a gene-specific primer as an adaptor, and M13R (GGATAACAAT TTCACACAGG) was also added to gene-specific primers (such as the primers for the *accD* gene, *matK* gene, and *rbcL* gene). The universal primers M13F and M13R

| Sample code | Materials name | Origin | Trib | Genus | Species |
|-------------|----------------|--------|------|-------|---------|
| A51 | Lanhuazi | China | Trib. Brassiceae Hayek | Raphanus L. | R. sativus |
| A52 | Luobozi (Ogu CMS) | Japan | Trib. Brassiceae Hayek | Raphanus L. | R. sativus |
| A53 | Eryuelan | China | Trib. Brassiceae Hayek | Orychophragmus Bunge | C. Violacea |
| A54 | Celezhahong | China | Trib. Brassiceae Hayek | Erca Mill | E. sativa |
| A55 | Yunjie | China | Trib. Arabideae DC | Rorippa | R. india |
| A56 | INIA 0863-66 | Spain | Trib. Brassiceae Hayek | Moricandia DC | M. arvensis |
| A57 | Banlangen | China | Trib. Lepidieae DC | Isatis | I. indigotica |
| A58 | Ziluolan | China | Trib. Matthioleae O.E.Schulz Matthiola | M. incana |
| A59 | Tri | Sweden | Trib. Arabideae DC | Arabidopsis | A. thaliana |
| A60 | KAS | Sweden | Trib. Arabideae DC | Arabidopsis | A. thaliana |
| A61 | Bsch | Sweden | Trib. Arabideae DC | Arabidopsis | A. thaliana |
| A62 | mr | Sweden | Trib. Arabideae DC | Arabidopsis | A. thaliana |
| A63 | Col | Sweden | Trib. Arabideae DC | Arabidopsis | A. thaliana |
| A64 | Zihuabangguoijie | China | Trib. Hesperideae Prantl | Sterigmostemum M.Bieb | S. matthioides |
| A65 | Yiguolan | China | Trib. Matthioleae O.E.Schulz Diptychocarpus Trautv | D. strictus |
| A66 | Choujie | China | Trib. Lepidieae DC | Coronopus J.G.Zimm | C. didymus |
| A67 | Dausanji | China | Trib. Sisymbrieae DC. | Sisymbrium L. | S. altissimum |
| A68 | Duoxingdasuanji | China | Trib. Sisymbrieae DC. | Sisymbrium L. | S. polymorphum |
| A69 | Xianghuajie | China | Trib. Hesperideae Prantl | Hesperis L. | H. trichosepala |
| A70 | Ququhua | China | Trib. Lepidieae DC | Iberis L. | I. amara |
| A71 | Guizhuxiang | China | Trib. Hesperideae Prantl | Chelanthus L. | Ch. cheiri |
| A72 | Hancai | China | Trib. Arabideae DC | Rorippa Scop. | R. india |
| A73 | Keshigaoyuanjie | China | Trib. Arabideae DC | Christolea Camb. | Ch. kashgarica |
| A74 | Doubancai | China | Trib. Arabideae DC | Nasturium R.Br | N. officinale |
| A75 | Lizjie | China | Trib. Matthioleae O. E. Schulz | Chorispora DC | Ch. tenella |
| A76 | Xianyunijie | China | Trib. Arabideae DC | Dimorphostemon Kitag | D. glandulosus |
| A77 | Ganxinnianzhuji | China | Trib. Sisymbrieae DC. | Tarularia (Coss)/O.E.Schulz | T. korolkowii |
| A78 | Qigangjie | China | Trib. Arabideae DC | Turritis L. | T. glabra |
| A79 | Sejie | China | Trib. Hesperideae Prantl | Malcolmia R.Br | M. africana |
| A80 | Suimijie | China | Trib. Arabideae DC | Cardamine L. | C. impatiens |
| A81 | Songlan | China | Trib. Lepidieae DC | Iberis L. | S. altissimum |
| A82 | Silengjie | China | Trib. Hesperideae Prantl | Goldbachia DC | G. laevigata |
| A83 | Xiaoyesuimijie | China | Trib. Arabideae DC | Cardamine L. | C. microzyga |
| A84 | Tuanshanjie | China | Trib. Alyssae Gren.et Godr Berteroa DC | B. incana |
| A85 | Taozundongxiejie | China | Trib. Alyssae Gren.et Godr Alyssum L. | A. linifolium |
| A86 | Xiangxiejie | China | Trib. Bresia Hayek | Corringia Adans | C. planisiliqua |
| A87 | Xiangxueqiu | China | Trib. Alyssae Gren.et Godr Lobularia Desv. | L. maritima |
| A88 | Yanjie | China | Trib. Sisymbrieae DC. | Theflunigella | T. salisugina |
| A89 | Xiaoyuqiyunijie | China | Trib. Sisymbrieae DC. | Camelina Crantz | C. microcarpa |
| A90 | Ziluolan | China | Trib. Matthioleae O.E.Schulz Matthiola R.Br | M. incana |

Table 1. Cont.

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Table 2. 90 accessions of cultivars of *B. napus*, and each of 3 accessions of *B. rapa* and *B. oleracea* used for ORG-EcoTILLING.

| Sample code | Materials name | Ploidy level | Genome         | Origin       |
|-------------|----------------|--------------|----------------|--------------|
| B1          | Midas          | 4 x          | AACC (n = 19)  | Canada       |
| B2          | Oro            | 4 x          | AACC (n = 19)  | Canada       |
| B3          | Major          | 4 x          | AACC (n = 19)  | France       |
| B4          | Primor         | 4 x          | AACC (n = 19)  | France       |
| B5          | Yaojin Rape    | 4 x          | AACC (n = 19)  | Italy        |
| B6          | Marnoo         | 4 x          | AACC (n = 19)  | Australia    |
| B7          | Ujbertadi      | 4 x          | AACC (n = 19)  | Hungary      |
| B8          | SavariA        | 4 x          | AACC (n = 19)  | Hungary      |
| B9          | Expander       | 4 x          | AACC (n = 19)  | Germany      |
| B10         | Ledos          | 4 x          | AACC (n = 19)  | Germany      |
| B11         | H47            | 4 x          | AACC (n = 19)  | Russia       |
| B12         | H51            | 4 x          | AACC (n = 19)  | Russia       |
| B13         | Janpol         | 4 x          | AACC (n = 19)  | Poland       |
| B14         | Start          | 4 x          | AACC (n = 19)  | Poland       |
| B15         | Mikado         | 4 x          | AACC (n = 19)  | England      |
| B16         | P20            | 4 x          | AACC (n = 19)  | England      |
| B17         | Lingot         | 4 x          | AACC (n = 19)  | England      |
| B18         | Wipot          | 4 x          | AACC (n = 19)  | Norway       |
| B19         | Regent         | 4 x          | AACC (n = 19)  | Canada       |
| B20         | Tower          | 4 x          | AACC (n = 19)  | Canada       |
| B21         | Shiralee       | 4 x          | AACC (n = 19)  | Australia    |
| B22         | Viking         | 4 x          | AACC (n = 19)  | Denmark      |
| B23         | Cobra          | 4 x          | AACC (n = 19)  | Germany      |
| B24         | Parter         | 4 x          | AACC (n = 19)  | Germany      |
| B25         | Falcon         | 4 x          | AACC (n = 19)  | Germany      |
| B26         | Nevin          | 4 x          | AACC (n = 19)  | France       |
| B27         | Samouran       | 4 x          | AACC (n = 19)  | France       |
| B28         | Roman-1        | 4 x          | AACC (n = 19)  | Netherlands  |
| B29         | Tornado        | 4 x          | AACC (n = 19)  | Sweden       |
| B30         | Legend         | 4 x          | AACC (n = 19)  | Sweden       |
| B31         | Grant          | 4 x          | AACC (n = 19)  | Sweden       |
| B32         | Celebra        | 4 x          | AACC (n = 19)  | Canada       |
| B33         | Triton         | 4 x          | AACC (n = 19)  | Canada       |
| B34         | Profit         | 4 x          | AACC (n = 19)  | Canada       |
| B35         | Startigh       | 4 x          | AACC (n = 19)  | Sweden       |
| B36         | Bounty         | 4 x          | AACC (n = 19)  | Sweden       |
| B37         | Garrison       | 4 x          | AACC (n = 19)  | Sweden       |
| B38         | Gcsunder       | 4 x          | AACC (n = 19)  | Germany      |
| B39         | Disamant       | 4 x          | AACC (n = 19)  | Germany      |
| B40         | Mar            | 4 x          | AACC (n = 19)  | Poland       |
| B41         | Star           | 4 x          | AACC (n = 19)  | Denmark      |
| B42         | Shengli Qinggen| 4 x          | AACC (n = 19)  | Shanghai, China |
| B43         | Jiuer Qinggen  | 4 x          | AACC (n = 19)  | Zhejiang, China |
| B44         | Hanfeng-1      | 4 x          | AACC (n = 19)  | Shanxi, China |
| B45         | Huayou-13      | 4 x          | AACC (n = 19)  | Wuhan, China |
| B46         | Aijia zao      | 4 x          | AACC (n = 19)  | Sichuan, China |
| B47         | Southeast-302  | 4 x          | AACC (n = 19)  | Sichuan, China |
| B48         | Yunyou-49      | 4 x          | AACC (n = 19)  | Yunnan, China |
| B49         | Qingyou-6      | 4 x          | AACC (n = 19)  | Qinghai, China |
| B50         | Nonglin-18     | 4 x          | AACC (n = 19)  | Japan        |
Table 2. Cont.

| Sample code | Materials name            | Ploidy level | Genome      | Origin          |
|-------------|---------------------------|--------------|-------------|-----------------|
| B51         | F01*J6 1-1                | 4 x          | AACC (n = 19) | Hubei, China    |
| B52         | Ganyou-5                  | 4 x          | AACC (n = 19) | Wuhan, China    |
| B53         | Zhongyou-821              | 4 x          | AACC (n = 19) | Wuhan, China    |
| B54         | Xiangyou-5                | 4 x          | AACC (n = 19) | Hunan, China    |
| B55         | Dong-Hae23                | 4 x          | AACC (n = 19) | Japan           |
| B56         | Ganpol                    | 4 x          | AACC (n = 19) | Zhejiang, China |
| B57         | Norin16                   | 4 x          | AACC (n = 19) | Japan           |
| B58         | Zheyouyou-2               | 4 x          | AACC (n = 19) | Zhejiang, China |
| B59         | Yuyou-2                   | 4 x          | AACC (n = 19) | Henan, China    |
| B60         | Zhongyoudijie-1           | 4 x          | AACC (n = 19) | Wuhan, China    |
| B61         | Qingyou-12                | 4 x          | AACC (n = 19) | Qinghai, China  |
| B62         | Qikuzheng                 | 4 x          | AACC (n = 19) | Japan           |
| B63         | Zhongshuang-4             | 4 x          | AACC (n = 19) | Wuhan, China    |
| B64         | ISN-705                   | 4 x          | AACC (n = 19) | India           |
| B65         | H0302                     | 4 x          | AACC (n = 19) | Hubei, China    |
| B66         | 2000-5                    | 4 x          | AACC (n = 19) | Hubei, China    |
| B67         | H9944                     | 4 x          | AACC (n = 19) | Hubei, China    |
| B68         | 05 Za-V2                  | 4 x          | AACC (n = 19) | Chongqing, China|
| B69         | 01 Za-654                 | 4 x          | AACC (n = 19) | Sichuan, China  |
| B70         | HY8                       | 4 x          | AACC (n = 19) | Jiangsu, China  |
| B71         | Youyan-10                 | 4 x          | AACC (n = 19) | Guizhou, China  |
| B72         | Qianyou-20                | 4 x          | AACC (n = 19) | Guizhou, China  |
| B73         | 6766                      | 4 x          | AACC (n = 19) | Hubei, China    |
| B74         | H0202                     | 4 x          | AACC (n = 19) | Hubei, China    |
| B75         | Zheyou-5002               | 4 x          | AACC (n = 19) | Zhejiang, China |
| B76         | Zhongyouza-2              | 4 x          | AACC (n = 19) | Hubei, China    |
| B77         | Za-839                    | 4 x          | AACC (n = 19) | Hunan, China    |
| B78         | Hongyou-3                 | 4 x          | AACC (n = 19) | Jiangsu, China  |
| B79         | Zashuang-5                | 4 x          | AACC (n = 19) | Henan, China    |
| B80         | 7633                      | 4 x          | AACC (n = 19) | Shanxi, China   |
| B81         | Qinyou-7                  | 4 x          | AACC (n = 19) | Shanxi, China   |
| B82         | Rape-23                   | 4 x          | AACC (n = 19) | Shanghai, China |
| B83         | Ganyou-4                  | 4 x          | AACC (n = 19) | Hubei, China    |
| B84         | Huayou-3                  | 4 x          | AACC (n = 19) | Hubei, China    |
| B85         | Huayou-8                  | 4 x          | AACC (n = 19) | Hubei, China    |
| B86         | ChuannongChangjiao         | 4 x          | AACC (n = 19) | Sichuan, China  |
| B87         | Chuanyou-7                | 4 x          | AACC (n = 19) | Sichuan, China  |
| B88         | Luzhou-5                  | 4 x          | AACC (n = 19) | Sichuan, China  |
| B89         | Nanyang-41                | 4 x          | AACC (n = 19) | Henan, China    |
| B90         | F11*J12 1-1               | 4 x          | AACC (n = 19) | Hubei, China    |
| B91         | Fenyang rape              | 2 x          | AA (n = 10)  | Shanxi, China   |
| B92         | Xishui rape               | 2 x          | AA (n = 10)  | Hubei, China    |
| B93         | Wenzhijiang Qixingjian     | 2 x          | AA (n = 10)  | Sichuan, China  |
| B94         | 2006 Holland-2            | 2 x          | CC (n = 9)   | Netherlands     |
| B95         | Chinese kale yellow/brown seeds | 2 x          | CC (n = 9)   | Guangdong, China|
| B96         | C2-7                      | 2 x          | CC (n = 9)   | Spain           |

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Discovery of Organelle Genomes DNA Polymorphisms

A

Reference DNA

Total DNA from plants

Query DNA

- PCR with target organelle gene primers with M13 attached to the 5' ends

B

- Pooling with 1:1

C

- Denature and re-anneal

D

- Digest with CEL I

E

- Denature

F

- Resolve on LI-COR gel

G

- Full length product
  - 0.9 kb
  - 0.5 kb
  - 0.4 kb
- Cleaved product labeled with forward primers
  - 700 channel image

- Full length product
  - Cleaved product labeled with reverse primers
  - 800 channel image
PCR amplification and mutation detection

PCR amplification and mutation detection followed the method of Barkley and Wang [58] with some modifications. For the accD, matK, and rbcL genes, we used a two-step approach to amplify DNA because the primer pairs carried the universal primers M13F and M13R. The first PCR amplification was performed in a 15 μL reaction volume using 60 ng DNA template, 1.5 μL Ex-Buffer, 0.2 mM dNTPs, 0.75 U Ex-Taq polymerase and 0.25 μL of each primer directly based on DNA sense strand, except a pair of primer against for \( \text{atp6} \) according to DNA antisense strand. All of the primers are listed in Table 3 and the positions of these primers are shown in Figure S1.

were labeled at the 5’ end with IRD 700 and IRD 800 separately (MWG Biotech, Inc., Ebersberg, Germany). Altogether, 7 pairs of primers were designed against for 3 genes (accD, matK and rbcL) based on DNA sense strand, except a pair of primer against for \( \text{atp6} \) according to DNA antisense strand. The primer pairs carried the universal primers M13F and M13R. The first PCR amplification was performed in a 15 μL reaction volume using 60 ng DNA template, 1.5 μL Ex-Buffer, 0.2 mM dNTPs, 0.75 U Ex-Taq polymerase and 0.25 μL of each primer directly based on DNA sense strand, except a pair of primer against for \( \text{atp6} \) according to DNA antisense strand. All of the primers are listed in Table 3 and the positions of these primers are shown in Figure S1.

**Table 3.** Primers used in the PCR amplification

| Taxon      | Genes | GenBank No. | Gene length | Primer name | Primer type | Primer sequence | PCR products length |
|------------|-------|-------------|-------------|-------------|--------------|------------------|---------------------|
| B. napus   | accD  | GQ861354    | 1470 bp     | accD-1      | Forward     | M13F-tgactattcactaatgtaatt | 974 bp              |
| B. rapa    | accD  | GQ861354    | 1470 bp     | accD-1      | Forward     | M13A1-Reverse | M13R-gttctattataagttcct | 798 bp              |
| B. oleracea| matK  | GQ861354    | 1575 bp     | matK-1      | Forward     | M13-M1-Forward | M13F-tgaggattagttcttg | 959 bp              |
| B. rapa    | matK  | GQ861354    | 1575 bp     | matK-1      | Forward     | M13-M1-Reverse | M13R-ttggagacacttagagtgcg | 948 bp              |
| B. oleracea| rbcL  | GQ861354    | 1440 bp     | rbcL-1      | Forward     | M13-R1-Forward | M13F-acacatattacactagag | 974 bp              |
| Cruciferae | accD  | AP000423    | 1476 bp     | CA          | Forward     | M13-CAFoward | M13F-tcaacgctgcttcttcatcttg | 1081 bp             |
| Cruciferae | rbcL  | GQ861354    | 1440 bp     | rbcL-1      | Forward     | M13-R2-Forward | M13F-caggattagagttcttg | 827 bp              |
| Cruciferae | atp6  | NM_126768.1 | 786 bp      | UPA         | Forward     | M13-CARReverse | M13F-ctttctttttttcttttacttg | 733 bp              |

| **Legend:**
| **PCR products length** | **PCR products length** |
|-------------------------|-------------------------|
| IRD700 Forward          | 700*-gaggattatagatcattcag | 733 bp |
| IRD800 Reverse          | 800*-atgctgtttctttctttcttctt | 733 bp |

Where 'IRD700' and 'IRD800' refer to the two wavelengths used in the gel imaging for detecting mutations. The primers are designed to amplify specific regions of the genes listed, and the PCR products are sequenced to identify any polymorphisms.

*Universal primers in this study included M13-IRD700 Forward and M13-IRD800 Reverse, and the corresponding primer sequences were 700*-cagcacgctgcttctttcttctt and 800*-ctttctttttttcttttacttg, respectively.

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plexes. The products were denatured and annealed in a thermal cycler, as follows: 99°C for 10 min, 70 cycles of 70°C for 20 sec, with a decrement of 0.5°C per cycle, and finally, 8°C for 5 min. A total of 30 μL of the processed PCR fragments was incubated in 2 μL of 10×CEL I buffer (10×CEL I buffer include 5 mL 1 M MgSO₄, 100 μL 10% Triton X-100, 5 mL 1 M Hepes (pH 7.4), 5 μL 20 mg/ml bovine serum albumen, 2.5 ml 2 M KCl, 37.5 ml ddH₂O). 0.2 μL purified CEL I extracted and purified according to the method described by Oleykowski et al (1998) [59] and 10 μL of heteroduplexes at 45°C for 30 min. CEL I cuts with partial efficiency, allowing the detection of multiple mismatches in a DNA duplex [48]. The CEL I digestion reaction was stopped with 5 μL of 0.225 mM EDTA. Sample purification was performed using Sephadex G50 (medium coarse) columns, and 2 μL of blue stop solution (MWG Biotech, Inc., Ebersberg, Germany) was added to each sample. Then, all of the samples were concentrated to a final volume of 2–3 μL for 60 min at 85°C. A total of 0.8 μL was loaded onto 6.5% polyacrylamide gels on a LI-COR 4300 DNA analyzer. To confirm the polymorphisms identified by ORG-EcoTILLING, each gel image of ORG-EcoTILLING was generated from two replicate runs. Because the fragment patterns for the two replicates were always concordant, we selected the better quality gel image for analysis. Furthermore, the accessions exhibiting polymorphism on the gels were randomly selected for a second round of amplification with the corresponding primer pairs using proofreading Ex-Taq polymerase. The PCR products were sequenced with an ABI 3730 and chromatograms checked to identify PCR or sequencing errors.

Mutation frequency and detection rate
A total of 141,120 bp, 151,200 bp and 138,240 bp were screened for the accD, matK and rbcL genes separately, which was...
calculated by the multiplication of the number of samples and the total length of the gene sequence. The mutation frequency was determined by dividing the total base pairs screened by the total mutants detected. The detection rate was calculated by the
analyses. The evolutionary distance \( D \) was computed using Kimura’s two-parameter method [62], and the trees tested with 1000 bootstrap replicates. The corresponding bootstrap values (>750) for each data partition were displayed in each node on the tree.

Results

Applicability of ORG-EcoTILLING in the discovery of chloroplast DNA polymorphism for inter- and intra-specific analysis of Brassica

A total of 90 B. napus samples and three each of B. rapa and B. oleracea were analyzed. The accD, matK and rbcL genes were amplified using two pairs of primers each. Amplified DNA fragments were of expected length for the three genes, as detected by agarose gel electrophoresis (Figure 2), and the DNA yield was suitable for ORG-EcoTILLING analysis. The analysis reliably located the position of mutated base pairs in the accD, matK and rbcL genes.

As an example, the IRD 700 and IRD 800 channels for the matK gene fragment ( Primer: M13-M2 Forward and M13-M2 reverse, Table S3) are shown in Figure 3. This demonstrates that mismatches were detected by CEL I and cleaved into two separate products, which were distinctly detected in both the IRD700 and IRD800 channels of the gel image. The combined lengths of the IRD700-labeled and IRD800-labeled cleaved fragments corresponded to the length of the PCR fragments (948 bp) in the matK gene. Similarly, clear gel images were obtained for the accD and rbcL genes, and the IRD700-labeled and the combined length of the IRD800-labeled cleaved fragments corresponded to the PCR fragments of the two genes (Table S1).

ORG-EcoTILLING detected nine, six and a single distinct mutated base pair positions in the accD, matK and rbcL genes, respectively, corresponding to 25, 25 and 19 mutated out of the 96 samples (Table S1). Two primer pairs were designed to amplify each of the three genes; their target regions were designated accD-1, accD-2, matK-1, matK-2, rbcL-1 and rbcL-2, respectively (Table 3). For the accD gene, most samples had one or two mutation points within accD-1, whereas there were five mutation points in accD-2, such as in samples B94, B95 and B96, B. oleracea, the ancestral parents of B. napus. In contrast, three or four mutation points were present in matK-2, but only one mutation point was found in matK-1. For the rbcL gene, a single mutation was observed in rbcL-1, and none in rbcL-2.

Interestingly, the three varieties of B. rapa possessed the same mutation positions as B. napus in the accD, matK and rbcL genes, respectively, whereas different mutation positions in the accD and matK genes were discovered in the three varieties of B. oleracea. For example, samples B94, B95 and B96 all had specific mutation points in the accD-1 gene fragment, as positions 409 and 871 in the IRD700 channel and five mutation positions in the accD-2 gene fragment, which were not observed in the B. napus and B. rapa varieties. Similarly, although the mutation positions in the matK-2 gene fragment were approximately the same in all mutation samples, only the three samples of B. oleracea presented one mutation position each in the matK-1 gene fragment. For the rbcL gene, the three samples of B. oleracea showed no mutation. Moreover, the samples mutated in the accD gene corresponded with those mutated in the matK gene, except sample B68. Similarly, the samples mutated in the rbcL gene were, without exception, distributed around the samples mutated of the accD and matK genes. Thus, our results effectively indicated that ORG-EcoTILLING could be effectively applied in chloroplast genes and that ORG-EcoTILLING could detect chloroplast DNA polymorphisms among the three species of Brassica, and very importantly among varieties of B. napus.
Application of ORG-EcoLLING for detecting SNPs within the accD gene across the Brassicaceae family

The key chloroplast gene accD was used to study the application of ORG-EcoTILLING in members of the Brassicaceae family (91 samples of different taxa). We designed the primers for the accD gene from a conservative region according to BLAST searches of different Brassicaceae taxa. The size of the accD fragment amplified by PCR was 827 bp in all samples.

Sufficient DNA was generated for subsequent analysis (Figure 4). Overall, 44 mutation points in the accD gene were detected in 44 plant samples (Table S2). Analysis of a subset of CEL 1 digests in the representative samples (Figure 5) revealed sample A61 and the reference sample A64 both lacked cleaved positions, whilst samples

Table 4. Analysis of ORG-EcoTILLING information in 700 and 800 channel from Figure 5.

| Mutation site No. | Geometric figure | IDY 700 | IDY 800 | Molecular Size (bp) | Sample code |
|-------------------|-----------------|--------|--------|---------------------|-------------|
| 1                 | blue triangle   | 73     | 754    | 827                 | A68         |
| 2                 | lime square     | 86     | 741    | 827                 | A65         |
| 3                 | lime square     | 87     | 740    | 827                 | A65         |
| 4                 | lime square     | 88     | 739    | 827                 | A65         |
| 5                 | red triangle    | 100    | 727    | 827                 | A65         |
| 6                 | orange ellipse  | 119    | 708    | 827                 | A65, A67, A68, A69 |
| 7                 | light green rectangle | 127 | 700    | 827                 | A65         |
| 8                 | orange rectangle| 134    | 693    | 827                 | A65         |
| 9                 | orange rectangle| 135    | 692    | 827                 | A65         |
| 10                | green rectangle | 144    | 683    | 827                 | A65, A68, A69 |
| 11                | blue rectangle  | 151    | 676    | 827                 | A65         |
| 12                | blue rectangle  | 152    | 675    | 827                 | A65         |
| 13                | yellow rectangle| 187    | 640    | 827                 | A65         |
| 14                | purple rectangle| 191    | 636    | 827                 | A65         |
| 15                | yellow ellipse  | 207    | 620    | 827                 | A68, A69    |
| 16                | turquoise circular | 225 | 602    | 827                 | A68, A69    |
| 17                | turquoise circular | 227 | 600    | 827                 | A69         |
| 18                | cyan ellipse    | 250    | 577    | 827                 | A68, A69    |
| 19                | cyan ellipse    | 251    | 576    | 827                 | A68, A69    |
| 20                | dark purple ellipse | 260 | 567    | 827                 | A65, A67, A68, A69 |
| 21                | turquoise ellipse| 283    | 544    | 827                 | A65         |
| 22                | emerald green ellipse | 305 | 522    | 827                 | A65         |
| 23                | blue ellipse    | 339    | 488    | 827                 | A65         |
| 24                | red ellipse     | 345    | 482    | 827                 | A65         |
| 25                | red ellipse     | 346    | 481    | 827                 | A65, A67, A68, A69 |
| 26                | purple rectangle| 372    | 455    | 827                 | A68, A69    |
| 27                | cyan rectangle  | 388    | 439    | 827                 | A65         |
| 28                | cyan rectangle  | 388    | 439    | 827                 | A68, A69    |
| 29                | cyan rectangle  | 389    | 438    | 827                 | A65, A67, A68, A69 |
| 30                | green circular  | 416    | 411    | 827                 | A65         |
| 31                | dark purple rectangle | 468 | 359    | 827                 | A65, A67    |
| 32                | dark purple rectangle | 469 | 358    | 827                 | A65, A67, A68, A69 |
| 33                | dark purple rectangle | 470 | 357    | 827                 | A65         |
| 34                | turquoise rectangle | 474    | 353   | 827                 | A65         |
| 35                | turquoise rectangle | 474    | 353    | 827                 | A67         |
| 36                | red rectangle   | 543    | 284    | 827                 | A65, A67    |
| 37                | blue rectangle  | 704    | 123    | 827                 | A65, A67, A68, A69 |
| 38                | emerald green rectangle | 713 | 114    | 827                 | A68, A69    |
| 39                | blue circular   | 755    | 72     | 827                 | A69         |
| 40                | red circular    | 781    | 46     | 827                 | A65         |

The sizes of the two fragments of IRD 700 and 800 indicated the accurate position of the mismatch. Descriptions of different color geometric figures were corresponding with that of Figure 5.

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Distinct SNPs were observed in the \textit{accD} gene of the Brassicaceae family; and only G to A transitions in the \textit{matK} gene were detected in sample A87, but no point mutations in other Trib. Brassicaceae accession. In contrast, abundant mutations of the \textit{atp6} gene were detected in Trib. Lepidieae and Trib. Sisymbrieae, the latter having eight SNPs in two plant samples. In addition, seven mutation points were detected in both of Trib. Hesperideae and Trib. Matthioleae, six SNPs in Trib. Arabideae and four polymorphic sites in Trib. Arabidaceae and four polymorphic sites in Trib. Alyssaceae. It should be noted that there are distinctly different in mutation sites within \textit{atp6} gene among different genus or species of the same tribes. ORG-\textit{EcoTILLING} images of seven samples (Figure 6) revealed that 13 nucleotide mutations were detected in the \textit{atp6} locus in samples A67, A76, A83 and A90, while samples A18, A24 and A32 lacking cleaved mutations were detected in the \textit{atp6} gene among these seven samples.

Figure 6. Images of ORG-\textit{EcoTILLING} gels of CEL I-digested products of a PCR fragment of \textit{atp6} gene obtained from each of the two fluorescent channels of the LI-COR 4300 DNA Analyzer. The intense bands (circled in different color geometric figures of IRD 700 channel and corresponding same color ones of IRD 800 channel) came from the product of CEL I-cleaved heteroduplexes. The sample in each lane from left to right was A24, A18, A32, A67, A76, A83 and A90, respectively, and sample A24 acted as the reference sample of \textit{atp6} gene for ORG-\textit{EcoTILLING}. Therefore, it was obvious that 13 polymorphism sites were detected in \textit{atp6} gene among these seven samples.

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A65, A67, A68 and A69 were characterized by 29, 9, 15 and 16 mutation sites, respectively. Furthermore, it was clear that the sizes of the 2 fragments produced for all mutation points indicated the accurate position of the mismatch, and thus the site of the mutation or nucleotide change. Detailed ORG-\textit{EcoTILLING} analysis for these four mutation samples is shown in Table 4. Distinct SNPs were observed in the \textit{accD} gene of the Brassicaceae family. For instance, these four mutation samples all shared with mutation site 6, 20, 25, 29, 32 and 37. However, sample A65 had 20 specific polymorphic sites which were not shared with other samples (Table 4). The results demonstrated that ORG-\textit{EcoTILLING} could not only be applied for detecting DNA polymorphisms in chloroplast genes in different tribes and genera, but also among and between species.

Application of ORG-\textit{EcoTILLING} for the detecting of SNPs within the \textit{atp6} gene across the Brassicaceae family

A key mitochondrial gene, \textit{atp6}, was chosen to study the application of ORG-\textit{EcoTILLING} in 91 different taxa of Brassicaceae. Initially, the primer for the \textit{atp6} gene was designed from a conserved region based on BLAST searches of different Brassicaceae taxa, with an expected PCR product length of 733 bp, which was confirmed in all 91 accessions by agarose gel electrophoresis (Figure 4). Analysis of all products of CEL I digests indicated that there were 17 mutation points distributed in 18 plant samples (Table 5). The distribution of the mutation points in the \textit{atp6} gene indicated that this \textit{atp6} gene was conserved amongst genera of Trib. Brassicaceae, since only three mutation points were observed in sample A87, but no point mutations in other Trib. Brassicaceae accession. In contrast, abundant mutations of the \textit{atp6} gene were detected in Trib. Lepidieae and Trib. Sisymbrieae, the former shared 11 mutation points among four plant accessions and the latter having eight SNPs in two plant samples. In addition, seven mutation points were detected in both of Trib. Hesperideae and Trib. Matthioleae, six SNPs in Trib. Arabidaceae and four polymorphic sites in Trib. Arabidaceae and four polymorphic sites in Trib. Alyssaceae. It should be noted that there are distinctly different in mutation sites within \textit{atp6} gene among different genus or species of the same tribes. ORG-\textit{EcoTILLING} images of seven samples (Figure 6) revealed that 13 nucleotide mutations were detected in the \textit{atp6} locus in samples A67, A76, A83 and A90, while samples A18, A24 and A32 lacking cleaved positions. Therefore, it was clearly shown that ORG-\textit{EcoTILLING} could be applied to the detection of mutations in a mitochondrial gene.

Validation of the accuracy of ORG-\textit{EcoTILLING} for the identification of cytoplasmic DNA Polymorphism

We validated the accuracy of ORG-\textit{EcoTILLING} by Sanger DNA sequencing. At the species level for the \textit{accD}, \textit{matK} and \textit{rbcL} genes in 96 samples of \textit{B. napus}, \textit{B. rapa} and \textit{B. oleracea}, mutation points were detected in 26, 26, and 19 samples, respectively. However, 70, 70 and 77 samples of \textit{B. napus} presented no mutation points in the corresponding genes, \textit{accD}, \textit{matK} and \textit{rbcL} by ORG-\textit{EcoTILLING}. According to the Sanger sequencing results, 116, 89 and 19 DNA polymorphisms were detected in \textit{accD}, \textit{matK}, and \textit{rbcL} genes, respectively, and the results of ORG-\textit{EcoTILLING} were entirely consistent with those of DNA sequencing results (Table 6). In addition, we calculated that the \textit{accD} gene in \textit{B. napus}, \textit{B. rapa} and \textit{B. oleracea} had approximately one mutation per 1.22 kb, compared with approximately one mutation per 1.70 kb and 7.28 kb in the \textit{matK} and \textit{rbcL} genes, respectively (Table 6). We then analyzed SNP classes based on the results of DNA sequencing. Two SNP classes of deletion and transition mutations were detected in the three genes, with the deletion type only presented in the \textit{accD} gene (in the form of six nucleotide base pair deletions). However, the majority of the transitions detected were C to A in the \textit{accD} gene; G to T, T to G and T to A in the \textit{matK} gene; and only G to A transitions in the \textit{rbcL} gene. All sequence variants detected by DNA sequencing were also detected by ORG-\textit{EcoTILLING}. Therefore, the detection rate of ORG-\textit{EcoTILLING} was 100%.
At the intertribal, intergeneric and interspecific levels, 40 and 13 mutation positions were detected by ORG-EcoTILLING from the total of 13 samples screened for the *accD* and *atp6* gene fragments, respectively (Figure 5 and 6). Although we identified only one type of mutation (base substitution) in the *accD* and *atp6* genes, there were abundant SNP types in these mutations (Table 7, Figure S2 and S3). There were 37, 15, 27 and 29 polymorphism sites (a total of 108 polymorphic sites) in *accD* gene among sample A65, A67, A68 and A69, respectively, and only 29, 9, 15 and 16 mutation sites (a total of 69 polymorphic sites) were discovered in these corresponding samples by ORG-EcoTILLING. Therefore, the detection rate within this gene was 64%. Two particular discrepancies primarily contributed to the low detection rate of ORG-EcoTILLING. Within the first 80 bp only one out of 13 polymorphic sites was detected. Additionally, between nucleotides 354 and 380 within *accD* gene in A65, A67, A68 and A69, respectively, and only 29, 9, 15 and 16 mutation sites (a total of 69 polymorphic sites) were discovered in these corresponding samples by ORG-EcoTILLING. Therefore, the detection rate within this gene was 64%. Two particular discrepancies primarily contributed to the low detection rate of ORG-EcoTILLING. Within the first 80 bp only one out of 13 polymorphic sites was detected. Additionally, between nucleotides 354 and 380 within *accD* gene in A65, A67, A68 and A69, there were 14 polymorphism sites within a range of 27 bases (Table 7, Figure S2), but only two of these were detected compared with the results of DNA sequencing. In contrast, all 13 polymorphic sites representing different types of SNPs within *atp6* gene fragments were detected using ORG-EcoTILLING (Table 7), thus the detection rate within this gene was 100%.

Very interestingly, we observed that the same polymorphic position detected by ORG-EcoTILLING revealed distinct transitions in different samples for the *accD* gene. For example, based on sequencing results, at position 365, base C mutated to base A, T and G among these samples, and for position 474, base A mutated to base C and T (Table 7, Figure S2). The latter was detected by ORG-EcoTILLING, while the former not.

Since this study involved quite distantly related accessions, it was necessary to verify whether the same polymorphic position detected by ORG-EcoTILLING represented identical transversion events within these samples. We therefore designed an experiment to validate the difference for the same polymorphic position discovered by ORG-EcoTILLING among species or higher taxa. Based on the results of ORG-EcoTILLING in the *matK*-2 fragment of *Brassica* species in Figure 3, we selected B76 mutated sample as the reference to mix with B26 (as the reference sample in Figure 3) and 19 mutated samples (also including B76, Figure 3), respectively. The result showed that there was no intense band in samples of B51, B64, B65, B66, B68, B69, B73, B74, B76, B79, B81, B91, B92 and B93, because these mutated samples had the same polymorphisms and positions as the reference sample B76. However, three polymorphic positions were discovered in the lane of B26, identical to those of the reference sample B76 (Figure 3). Interestingly, the lanes for B60, B90, B94, B95 and B96 all showed one polymorphic position (Figure 7a), primarily due to the fact that these samples held the special polymorphism which the reference sample B76 did not share with (Figure 3). Thus, the result clearly demonstrated that the same polymorphic position detected by ORG-EcoTILLING revealed the same polymorphism between *Brassica* intraspecies.

Plants from different genera or tribe shared polymorphisms in *accD* and/or *atp6* whereas they did not share with other accessions of the same species. For example, it was obviously not the case for position 474 in *accD* between samples A65 and A67 (Figure S2). Therefore, we designed an ORG-EcoTILLING experiment to test them by using one of the three samples of A64, A65 and A67 as reference with the others. The result revealed the presence of polymorphisms when A65 was mixed with A67, suggesting that distinct polymorphisms were located at the same position (Figure 7b).

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**Table 5.** Band analysis of *atp6* gene in the family of Brassicaceae identified by ORG-EcoTILLING.

| Mutated sample code | 134 | 140 | 240 | 296 | 305 | 314 | 338 | 351 | 368 | 465 | 512 | 533 | 625 | 637 | 647 | 653 | 663 |
|---------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A56                 | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| A58                 | +   | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |
| A60                 |     | +   |     | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |     |
| A61                 |     |     | +   | +   | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |
| A67                 | +   |     | +   | +   | +   |     |     | +   |     |     |     |     |     |     |     |     |     |
| A68                 |     | +   |     |     |     |     |     |     |     | +   |     |     |     |     |     |     |     |
| A70                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| A71                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| A72                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| A73                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| A75                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| A76                 | +   | +   | +   | +   | +   | +   | +   | +   | +   |     |     |     |     |     |     |     |     |
| A79                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| A82                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| A83                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| A87                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| A88                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| A90                 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

*"++" indicated that there was an intense band visualized by denaturing polyacrylamide gel electrophoresis with the LI-COR DNA analyzer.*

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### Table 6. SNP mutation types and band analysis of *B. napus* varieties identified by ORG- EcoTILLING and DNA sequencing.

| Sample code | accD mutation position | matK mutation position | rbcl. mutation position |
|-------------|-------------------------|------------------------|-------------------------|
|             | 216 (G/T)               | 560-565 (AAAGTG)      | 934 (G/A)              |
|             | 335 (T/A)               | 644 (A/C)              | 1031-1032 (T/T/AA)     |
|             | 797 (C/T)               | 858 (G/A)              | 1132 (G/T)             |
|             | 1058 (T/G)              | 1177 (T/C)             | 66 (G/A)               |
| B11         | +                       | +                      | +                       |
| B12         | +                       | +                      | +                       |
| B14         | +                       | +                      | +                       |
| B25         | +                       | +                      | +                       |
| B27         | +                       | +                      | +                       |
| B29         | +                       | +                      | +                       |
| B32         | +                       | +                      | +                       |
| B45         | +                       | +                      | +                       |
| B51         | +                       | +                      | +                       |
| B60         | +                       | +                      | +                       |
| B64         | +                       | +                      | +                       |
| B65         | +                       | +                      | +                       |
| B66         | +                       | +                      | +                       |
| B68         | +                       | +                      | +                       |
| B69         | +                       | +                      | +                       |
| B73         | +                       | +                      | +                       |
| B74         | +                       | +                      | +                       |
| B76         | +                       | +                      | +                       |
| B79         | +                       | +                      | +                       |
| B81         | +                       | +                      | +                       |
| B90         | +                       | +                      | +                       |
| B91         | +                       | +                      | +                       |
| B92         | +                       | +                      | +                       |
| B93         | +                       | +                      | +                       |
| B94         | +                       | +                      | +                       |
| B95         | +                       | +                      | +                       |
| B96         | +                       | +                      | +                       |
| Total       | 116                     | 23                     | 27                      |

**Discovery of Organelle Genomes DNA Polymorphisms**
Phylogenetic relationships of chloroplast genes among three *Brassica* species

In order to exploit the phylogenetic relationship among the different *Brassica* species based on the polymorphism results by ORG-EcoTILLING, a phylogenetic tree was constructed using 288 chloroplast genes sequences. Ninety DNA sequences representing each of *accD*, *matK* and *rbcL* in *B. napus*, and three sequences of each of three chloroplast genes in *B. oleracea* and *B. rapa* were analysed. Based on the results no mutation was found to have an identical DNA sequence as that of the reference sample, and this greatly reduced the samples available for DNA sequencing. For example, 27 samples were sequenced for *accD* and *matK* (including the reference), and 20 samples for *rbcL* (including the reference). Therefore, it was possible for us to analyze DNA polymorphisms in a large number of samples, merely through the strategy of combining ORG-EcoTILLING results with existing sequences data. The results revealed three distinct classes (I, II and III) within the genus (Figure 8). The three *B. oleracea* accessions were distinctly diverged from both *B. napus* and *B. rapa* accessions, and the majority of *B. napus* clustered with three accessions of *B. rapa*. However, the three accessions of *B. napus*, B14, B60 and B90 clustered in a clade (II), distinct from other accessions.

In conclusion, our results demonstrate that there are obvious differences in the detection rate of cytoplasmic DNA polymorphisms by ORG-EcoTILLING within the Brassicaceae family. *B. napus* interspecific and intraspecific levels, 100% of mutations were detected in SNPs and INDELs in the *accD*, *matK* and *rbcL* genes. However, this method could not detect all mutation events in the *accD* gene at the genus level in the taxa investigated. In contrast, the detection rate within the *atp6* gene was 100% accurate among the Brassicaceae family. Moreover, the information from ORG-EcoTILLING revealed genetic variations within the species of *B. napus*.

Discussion

We have demonstrated that a CEL I-based heteroduplex cleavage strategy, which was originally developed for TILLING and EcoTILLING [48,49,63], can be successfully applied to the discovery of chloroplast and mitochondrial DNA polymorphisms, especially at the inter- and intraspecific levels. TILLING and EcoTILLING have been proven to be inexpensive and efficient technologies for the discovery of DNA polymorphisms within specific genes. By applying these methods, much progress has been achieved. However, all existing studies have focused on detecting genetic variation within nuclear genomes of different eukaryotic organisms [44–47,64–67]. The results of the present study suggested that the development of ORG-EcoTILLING will provide a high-throughput and cost-effective alternative to the current state-of-the-art techniques for studying chloroplast and mitochondrial genomes. Application of new methods in phylogenetic and ecological studies usually leads to important progress in a range of fields. For example, using chloroplast microsatellites, much progress has been achieved in recent decades in investigations of population genetics, crop plant evolution and domestication and phylogenetics [14]. As ORG-EcoTILLING has several important advantages over chloroplast microsatellites, we can expect widespread application of this new tool in chloroplast and mitochondrial genome studies, with more informative outcomes.

Compared with existing techniques, this method requires only a minimal amount of plant tissue or DNA and avoids the laborious experimental procedures associated with cpDNA and mtDNA purification, DNA enzymatic digestion and Southern hybridiza-
Table 7. Comparison of efficiency in different types of SNPs in accD and atp6 gene detected by ORG-EcoTILLING and DNA sequencing.

| positions of polymorphisms | SNPs detected by sequencing | SNPS detected by ORG-EcoTILLING | Detection rate (%) |
|---------------------------|----------------------------|---------------------------------|--------------------|
|                           | A64* A65 A67 A68 A69      | A64* A65 A67 A68 A69            |                    |
| accD gene                 |                            |                                 |                    |
| 56                        | C T T T T              | NP  -  -  -  -    | 0                 |
| 63                        | A A A G G            | NP NP  -  -  -    | 0                 |
| 73                        | G G G A              | NP NP  NP + NP   | 100               |
| 74                        | T C C C C            | NP  -  -  -  -    | 0                 |
| 81                        | T T T G G            | NP NP  -  -  -    | 0                 |
| 86                        | G A G G              | NP NP  NP NP NP  | 100               |
| 87                        | A C A A A            | NP NP  NP NP NP  | 100               |
| 88                        | T A T T T            | NP NP  NP NP NP  | 100               |
| 100                       | G T G G G            | NP NP  NP NP NP  | 100               |
| 109                       | G G G A              | NP NP  -  -  -    | 0                 |
| 119                       | A G G G G            | NP  +  +  +  +    | 100               |
| 127                       | A G A A A            | NP NP  NP NP NP  | 100               |
| 134                       | C A C C C            | NP NP  NP NP NP  | 100               |
| 135                       | G T G G G            | NP NP  NP NP NP  | 100               |
| 144                       | C A C A A            | NP NP  +  +  +    | 100               |
| 151                       | A C A A A            | NP NP  NP NP NP  | 100               |
| 152                       | C T C C C            | NP NP  NP NP NP  | 100               |
| 187                       | G T G G G            | NP NP  NP NP NP  | 100               |
| 191                       | A G A A A            | NP NP  NP NP NP  | 100               |
| 207                       | A A A G G            | NP NP  +  +  +    | 100               |
| 225                       | C C C G G            | NP NP  +  +  +    | 100               |
| 227                       | A A A A G            | NP NP  NP NP NP  | 100               |
| 250                       | G G G C C            | NP NP  NP NP NP  | 100               |
| 251                       | T T T C C            | NP NP  NP NP NP  | 100               |
| 260                       | A G G G G            | NP  +  +  +  +    | 100               |
| 266                       | C T C T T            | NP  -  -  -  -    | 0                 |
| 283                       | C G C C C            | NP NP  NP NP NP  | 100               |
| 305                       | T C T T  T           | NP  +  NP NP NP  | 100               |
| 339                       | C A C C C            | NP NP  NP NP NP  | 100               |
| 345                       | A C A A A            | NP NP  NP NP NP  | 100               |
| 346                       | A G G G G            | NP  +  +  +  +    | 100               |
| 354                       | C A C C C            | NP  -  NP NP NP  | 0                 |
| 365                       | C A T G G            | NP  -  -  -  -    | 0                 |
| 368                       | T T T C C            | NP NP  NP NP NP  | 100               |
| 372                       | G G G A A            | NP NP  +  +  +    | 100               |
| 380                       | G T T T T            | NP  -  -  -  -    | 0                 |
| 388                       | C T C A A            | NP NP  NP NP NP  | 100               |
| 389                       | T G G G G            | NP  +  +  +  +    | 100               |
| 395                       | A A A G G            | NP NP  -  -  -    | 0                 |
| 408                       | G G G A A            | NP NP  -  -  -    | 0                 |
| 416                       | A C A A A            | NP NP  NP NP NP  | 100               |
| 449                       | C C C T T            | NP NP  NP NP NP  | 100               |
| 450                       | C A C C C            | NP  -  NP NP NP  | 0                 |
| 468                       | C A A C C            | NP  +  NP NP NP  | 100               |
| 469                       | G A A A A            | NP  +  +  +  +    | 100               |
| 470                       | G T G G G            | NP NP  NP NP NP  | 100               |
| 474                       | A C T A A            | NP NP  NP NP NP  | 100               |
The use of genomic DNA is one of the key advantages of the successful application of chloroplast microsatellites. We can apply this method to survey natural variation in a region of interest accurately and affordably, which is extremely important for chloroplast and mitochondrial genome research because these genomes share the common characteristic of slow nucleotide substitution rates. If more regions are assessed, the information content will rise accordingly. Point mutations are unevenly distributed in plant chloroplast genomes. Magee et al. (2010) found a region of chloroplast DNA in plants related to the sweet pea (Lathyrus) in which the local point mutation rate is at least 20 times higher than elsewhere in the same molecule [68]. In this study, it was found that the accD, matK and rbcL genes present different point mutation rates among different regions in Brassica species (Table 7), which suggests that more regions need to be studied. ORG-EcoTILLING can efficiently detect rare SNPs and INDELs in specific chloroplast genes with high throughput (including coding regions and non-coding regions). Using a Li-COR gel analyzer, 96 samples can be screened in a single gel run using 1:1 reference and query pooled samples, and the efficiency is much higher than that of DNA sequencing.

Under the experimental conditions used here, the endonuclease CEL I cuts with partial efficiency, allowing the detection of multiple mismatches in a DNA duplex [48,69]. We identified both SNPs and small INDELs in chloroplast and mitochondrial gene fragments over a roughly 800-bp window (1 kb-2×100 bp, the terminal noisy regions), similar to EcoTILLING for nuclear genes [48]. However, the degree of detection accuracy varies at different taxon levels. For example, at inter- and intraspecific levels, 10, six and one mutation sites were discovered by ORG-EcoTILLING in the accD, matK and rbcL genes, respectively, and the detection rates of these chloroplast genes were all 100% (Table 6), which corresponded with the results of DNA sequencing. Furthermore, at higher taxonomic levels (among tribes and genera in Brassicaceae), the total detection rate of the chloroplast gene accD was only 64%, whereas it was 100% in the mitochondrial gene atp6 (Table 7).

We found that as the number of mutations detected per fragment increases, the scoring and tracking of cleaved fragments becomes more difficult. The lower accuracy at high taxonomic levels could be attributed to higher numbers of polymorphisms occurring among distant samples at intertribal or intergeneric levels, as in the case of the accD gene, from nucleotide 354 to 380 of the accD gene in A65, A67, A68 and 69, there were 14 polymorphism sites within a range of 27 bases, but only two of these were detected compared with the results of DNA sequencing.

| positions of polymorphisms | SNPs detected by sequencing | SNPs detected by ORG-EcoTILLING | Detection rate (%) |
|----------------------------|-----------------------------|-------------------------------|--------------------|
|                            | A64* | A65 | A67 | A68 | A69 | A64* | A65 | A67 | A68 | A69 | A64* | A65 | A67 | A68 | A69 |
| 522                        | A G  | A A | A A | A A | NP | -   | NP | NP | NP | NP | 0   |
| 543                        | T C  | C C | T T | T T | NP | +   | NP | NP | NP | NP | 100 |
| 704                        | A G  | G G | G G | G G | NP | +   | +  | NP | NP | NP | 100 |
| 707                        | G G  | C G | G G | G G | NP | NP | -  | NP | NP | NP | 0   |
| 713                        | T T  | T T | A A | A A | NP | NP | NP | NP | +  | NP | 100 |
| 746                        | C C  | T C | C C | C C | NP | NP | -  | NP | NP | NP | 0   |
| 755                        | T T  | T T | T T | T T | NP | NP | NP | NP | NP | NP | 100 |
| 781                        | G T  | G G | G G | G G | NP | NP | NP | NP | NP | NP | 100 |

Total mutation sites: 108
Average Detection rate (%): 64

| positions of polymorphisms | SNPs detected by sequencing | SNPs detected by ORG-EcoTILLING | Detection rate (%) |
|----------------------------|-----------------------------|-------------------------------|--------------------|
|                            | A24* | A67 | A76 | A83 | A90 | A24* | A67 | A76 | A83 | A90 |
| 134                        | G T  | T T | G G | G G | NP | +   | NP | NP | NP | NP | 100 |
| 240                        | G C  | C C | C C | G G | NP | +   | +  | NP | NP | NP | 100 |
| 296                        | T T  | T C | T C | T C | NP | NP | NP | NP | NP | NP | 100 |
| 305                        | T C  | C C | T T | T T | NP | +   | +  | NP | NP | NP | 100 |
| 314                        | T G  | G G | G G | T T | NP | NP | +   | NP | NP | NP | 100 |
| 338                        | A A  | A A | A A | A G | NP | NP | NP | NP | NP | +  | 100 |
| 351                        | C G  | G G | G G | C C | NP | NP | NP | NP | NP | NP | 100 |
| 512                        | T T  | T T | T C | T C | NP | NP | NP | NP | NP | NP | 100 |
| 533                        | T T  | T T | T T | T T | NP | NP | NP | NP | NP | NP | 100 |
| 625                        | T C  | C C | C C | C C | NP | NP | NP | NP | NP | NP | 100 |
| 637                        | G G  | G G | G G | A A | NP | NP | NP | NP | NP | NP | 100 |
| 647                        | A A  | A A | A A | T T | NP | NP | NP | NP | NP | NP | 100 |
| 653                        | C A  | A A | A A | C C | NP | NP | NP | NP | NP | NP | 100 |

Average Detection rate (%): 100

*indicated that the reference sample was A64 and A24 in accD gene and atp6 gene, respectively. “+” indicated that there was an intense band visualized by ORG-EcoTILLING. “++” indicated that there was no intense band detected by ORG-EcoTILLING. “NP” indicated no polymorphism.

Table 7. Cont.
Therefore, overestimating the number of mismatches decreased the resolution and signal intensity in the CEL I analysis, which was also found in the case of the PIF2-2 haplotype [48]. Moreover, the accuracy of this method may be reduced in extreme situations such as where multiple mutations occur in a very short region. In this study, only one of 13 polymorphic sites in the accD gene was discovered within the first 80 bp (Table 7).

Recently, increasingly complete chloroplast and mitochondrial genome sequences of many species of animals, plants and microbes have been obtained by applying next-generation DNA sequencing technologies [56,70–72]. These complete genome sequences provide important references, but they are still not sufficient for conducting phylogenetic analysis and functional studies of chloroplast and mitochondrial genes, in which more information for individuals in populations is needed. The increased number of polymorphisms screened in organelle DNA is particularly advantageous for evolutionary and ecological studies because the level of DNA polymorphism at inter- and intraspecific levels is generally very low, and our results indicate that ORG-EcoTILLING is ideally suited for the high-throughput, accurate discovery of rare chloroplast and mitochondrial DNA polymorphisms. Therefore, ORG- EcoTILLING is a good alternative for assessment of organelle genome variation.

The most important and attractive application of this method is to conduct genome-wide assessments of chloroplast and mitochondrial DNA diversity within a population at the inter- and intraspecific level, which is particularly important for providing a better understanding of the evolution of organelle genomes and the evolution and ecology of species of interest. By using a gene overlapping strategy, we will be able to construct haplotype maps of chloroplast and mitochondrial genomes efficiently. At present, only a few haplotype maps of the nuclear genomes of humans, Arabidopsis and maize have been constructed [73–75]. Unlike the nuclear genome, because the polymorphism level of organelle genomes are much lower, and the recombination is much rarer, it is unnecessary to sequence genome of all individuals of a species for the purpose of haplotype map construction. A more efficient way to do this is to use the ORG-EcoTILLING strategy. The size of the chloroplast genome in plants is 120–200 kb [11]. A single EcoTILLING run generally covers a 1-kb target region; considering overlap and terminal noisy regions. With approximately 200 runs, we can expect to obtain genome-wide coverage of 96 samples and observe the full spectrum of variation. Organelle haplotype
maps will provide global documented information on inter- and intraspecific variations, which will greatly facilitate studies of the evolution, domestication, population genetics and gene structure and organization.

The data from ORG-EcoTILLING could be applied to analyze phylogenetic relationships. Our study demonstrated that the three *B. oleracea* accessions were distinctly diverged from both *B. napus* and *B. rapa* accessions, while the majority of *B. napus* clustered with three accessions of *B. rapa*. The probable reason is that most accessions of *B. napus* originated from the cytoplasm of *B. rapa*, suggesting that *B. rapa* was a much more likely maternal progenitor for *B. napus* than *B. oleracea*, which was consistent with previous reports [76,77]. Moreover, genetic variations were also observed within the *B. napus*, such as B14, B60 and B90, which were diverged from other accessions (Figure 8), most likely due to the multiple origins or evolution in the relatively recent domestication and modern breeding *B. napus*.

**Figure 8.** Phylogenetic tree was constructed based on the integrating data of the three chloroplast genes (*accD*, *matK* and *rbcL*) in *Brassica* species. A total of *accD*, *matK* and *rbcL* sequences of 90 accessions of *B. napus* and each of 3 accessions of *B. rapa* and *B. oleracea* were collected for analysis. The tree was constructed by the neighbour-joining method and bootstrap values above 750 from 1000 resamplings were shown for each node. The code in the tree corresponded to the locality code of Table 2. M represented 69 no mutation samples in *B. napus*. The bar corresponded with 100 substitutions per site.

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Although this study focused on plant chloroplast and mitochondrial DNA polymorphisms, the method developed here is also likely to be applicable to human and animal mitochondrial genomes. The high-throughput 454 method was recently used to sequence the complete human mtDNA genome of 109 individuals from the Philippines, and ~55-fold coverage was achieved, generating <1% missing data per sequence [78]. If ORG-EcoTILLING is proven to be applicable to human and animal mitochondrial genomes, as the size of human and animal mitochondrial genomes is approximately 16 kb [79], additional genome-wide investigations of mitochondrial genomes will become practical not only in humans, but also in many animal species.

There are some limitations to ORG-EcoTILLING. For example, the method does not directly generate sequence information about the detected mutations, and one representative sample among many individuals sharing identical DNA haplotypes must be sequenced to acquire sequence information for the subset. In this case, complementary DNA sequencing would be needed for the extraordinary region.

The rapid development of NGSts promises the possibility of global studies of chloroplast and mitochondrial genomes at population levels. However, ORG-EcoTILLING is ideally suited to screening large numbers to survey populations and diversity collections in chloroplast and mitochondrial genomes, thus reducing samples for which sequencing would provide more detailed information. Therefore, ORG-EcoTILLING represents an alternative new tool for high-throughput, high-resolution analysis of organelle genomes.

Supporting Information

Figure S1 A schematic representation of the position of the primers on accD, matK, rbcL and atp6 genes. Two pairs of primers were designed in accD, matK and rbcL of 96 Brassica species, whereas only one pair of labeled primer was designed in accD and atp6 genes of 91 accessions from Cruciferae. Black boxes showed the genes, and detailed information on each primer was shown in Table 3.

References

1. Azam A, Paul J, Sehgal D, Prasad J, Bhattacharya S, et al. (1996) Identification of novel genes from Entamoeba histolytica by expressed sequence tag analysis. Gene 181: 113–116.
2. Botstein D, White RL, Skolnick M, Davis RW (1980) Construction of Agenetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 32: 314–331.
3. Feral J (2002) How useful are the genetic markers in attempts to understand and manage marine biodiversity? J Exp Mar Biol Ecol 268: 121–145.
4. Liu Z, Corder J (2004) DNA marker technologies and their applications in aquaculture genetics. Aquaculture 238: 1–37.
5. Ryman N, Utter F (1987) Population genetics and fishery management. Seattle: University of Washington Press.
6. Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic acids Res 18: 7213–7218.
7. Palmer JD, Herbon LA (1988) Plant mitochondrial DNA evolved rapidly in a family of plants (Cucurbitaceae). Cell 25: 793–803.
8. Palmer JD (1985) Evolution of chloroplast and mitochondrial DNA in plants and proteins (eds. Nei M, Koehn RK), Sinauer, Sunderland MA. pp. 62–88.
9. Birky C (1995) Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. Proc Natl Acad Sci 92: 11331–11338.
10. Dumolin S, Demesure B, Petit R (1995) Inheritance of chloroplast and mitochondrial genomes in Chenopodium silvaticum investigated with an efficient PCR method. Theor Appl Genet 91: 1253–1256.
11. Radetzky R (1996) Analysis of mitochondrial DNA and its inheritance in Populus. Curr Genet 18: 429–434.
12. Rajora O, Danicik B (1992) Chloroplast DNA inheritance in Populus. Theor Appl Genet 84: 280–285.
13. Hofkinson T, Chase M, Takaishi C, Leitch I, Bennett M, et al. (2002) The use of DNA sequencing (ITS and trnL-F), AFLP, and fluorescent in situ hybridization to study allopolyploid Miscanthus (Poaceae). Am J Bot 89: 279–286.
14. Vos P, Hogers R, Bleeker M, Reijans M, Wagten E, et al. (1995) AFLP: a new fingerprinting technique. Plant Mol Biol Rep 13: 103–115.
15. Provan J, Powell W, Hollingworth P (2001) Chloroplast microsatellites: new tools for studies in plant ecology and evolution. Trends Ecol Evol 16: 142–147.
16. Fox JG, Hobbs PB (1988) Mitochondrial DNA sequence polymorphisms in a land-plant species: a mechanism for monitoring hybridization. Theor Appl Genet 77: 620–624.
17. Fox JG, Hobbs PB (1988) Mitochondrial DNA sequence polymorphisms in a land-plant species: a mechanism for monitoring hybridization. Theor Appl Genet 77: 620–624.
25. Vedel F, Quetier F, Doshi F, Doussainh G (1978) Study of wheat phylogeny by coRI analysis of chloroplastic and mitochondrial DNAs. Plant Sci Letters 13: 97–102.

26. Fernandes CA, Gimia C, Pereira I, Tenreiro R, Bradford MW, et al. (2008) Species-specific mitochondrial DNA markers and polymerase chain reaction-restriction fragment length polymorphism for domestic and wild species identification. Afr J Biotechnol 5: 1588–1593.

27. Malina A, Gwakia P, Balthazar S, Wasser S, Mutotya B (2006) The potential of mitochondrial DNA markers and polymerase chain reaction-restriction fragment length polymorphism for domestic and wild species identification. Afr J Biotechnol 5: 1588–1593.

28. Wu F, Zhang Z, Dai H, Zhang Y, Chang L (2006) Genetic relationship of some Crataegus spp. (Hawthorn) revealed by chloroplast DNA PCR-RFLP. J Biotechnol 136: S103.

29. Li YP, Yang BS, Wang H, Xia RX, Wang L, et al. (2009) Mitochondrial DNAAnalysis reveals a low nucleotide diversity of Caialus japonica in China. Afr J Biotechnol. 8: 2707–2712.

30. Ursin VM, Becker CK, Shewmaker CK (1993) Cloning and nucleotide sequence of a tobacco chloroplast translational elongation factor, EF-Tu. Plant Physiol 101: 311–333.

31. Cubas P, Pardo C, Tahiri H (2005) Genetic variation and relationships among Ulex (Fabaceae) species in southern Spain and northern Morocco assessed by chloroplast microsatellite (cpSSR) markers. Ann Bot 96: 2031.

32. Jakobsson M, Sall T, Lind-Hallden C, Hallgren C (2007) Evolution of the chloroplast mononucleotide microsatellites in Arabidopsis thaliana. Theor Appl Genet 114: 223–235.

33. Chase M, Solins D, Olmstead R, Morgan D, Les D, et al. (1993) Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene rbcL. Ann Bot 64: 501–519.

34. Steele K, Vilgalys R (1994) Phylogenetic analyses of Polemoniaceae using chloroplast microsatellite (cpSSR) markers. Am J Bot 92: 2031.

35. Nagano Y, Matsuno R, Sasaki Y (1991) Sequence and transcriptional analysis of the genome of Nymphaea alba: whole-genome analyses and the problem of chloroplast microsatellite (cpSSR) markers. Am J Bot 92: 2031.

36. Fukami H, Omori M, Hatt a M (2000) Phylogenetic relationships in the coral family Acroporidae, reassessed by inference from mitochondrial genes. Zool Sci 17: 689–696.

37. Ratnasingham S, Hebert PDN (2007) BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). Mol Ecol Notes 7: 355–364.

38. Metzker ML (2010) Sequencing technologies—the next generation. Nat Rev Genet 11: 31–46.

39. Kristensen V, Kelefiotis D, Kristensen T, Borresen-Dale AL (2001) High-throughput sequencing of complete human mtDNA genomes from the Musa gene pool by EcoTILLING. Genome Res 11: 311–319.

40. Tsuchihashi Z, Dracopoli NC (2002) Progress in high throughput SNP genotyping applications for reverse genetics. Curr Opin Plant Biol 8: 211–215.

41. Goremykin V, Hirsch-Ernst K, Wohlleben W, et al. (2004) De novo variation in life-history traits and complex trait evolution in Myrtaceae trees. Evolution 58: 2477–2483.

42. Schranz ME, Osborn TC (2004) De novo variation in life-history traits and complex trait evolution in Myrtaceae trees. Evolution 58: 2477–2483.

43. Henikoff S, Comai L (2000) Single-Nucleotide Mutations for Plant Functional Genomics. Plant Biotechnology. Teixeira da Silva JA, (ed), Global Science Books, Ltd., London, Vol I, pp. 476–482.

44. Murray M, Thompson W (1980) Rapid isolation of high molecular weight plant DNA. Nucleic acids res 8: 4361–4363.

45. Colbert T, Till B, Tompa R, Reynolds S, Steine M, et al. (2001) High-throughput sequencing of complete human mtDNA genomes from the Musa gene pool by EcoTILLING. Genome Res 11: 311–319.

46. Song K, Lu P, Tang K, Osborn T (1993) Rapid genome change in synthetic polyploids of Brassica and its implications for polyploid evolution. Proc Natl Acad Sci 92: 7719–7723.

47. Olyekowski CA, Bronson Mullins CR, Godwin AK, Yeung AT (1998) Mutation detection using a novel plant endonuclease. Nucleic Acids Res 26: 4597–4602.

48. Thompson DJ, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.

49. Stemple DJ (1997) An alternating least-squares approach to inferring phylogenies from pairwise distances. Syst Zool 46: 101–111.

50. Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111–120.

51. Till BJ, Reynolds SH, Greene EA, Codomo CA, Enns LC, et al. (2003) Large-scale discovery of induced point mutations with high-throughput TILLING. Genome 46: 524–530.

52. Nieto C, Piron F, Damais M, Marco C, Moriones E, et al. (2007) EcoTILLING for the identification of allelic variants of melon. J Exp Bot 58: 3181–3184.

53. Vedel F, Quetier F, Doshi F, Doussainh G (1978) Study of wheat phylogeny by coRI analysis of chloroplastic and mitochondrial DNAs. Plant Sci Letters 13: 787–786.

54. Colbert T, Till B, Tompa R, Reynolds S, Steine M, et al. (2001) High-throughput screening for induced point mutations. Plant Physiol 126: 480–484.

55. McCallum C, Comai L, Greene E, Henikoff S (2000) Targeting induced local lesions in genomes (TILLING) for plant functional genomics. Plant Physiol 123: 439–442.

56. Tsai H, Howell T, Nich ter R, Misurian V, Watson B, et al. (2011) Discovery of rare mutations in populations: TILLING by sequencing. Plant Physiol 156: 1257–1268.

57. Appel O, Al-Shelah I (2003) Cruciferae. In The families and genera of vascular plants (Kubitzki K, Bayer C, eds). Heidelberg: Springer-Verlag Berlin, pp. 73–137.

58. Baum D, Yoon H, Oldham R (2005) Molecular evolution of the transcription factor LEAFY in Brassicaceae. Mol Phylogenet Evol 37: 1–14.

59. Schranz M, Osborn T (2004) De novo variation in life-history traits and responses to growth conditions of resynthesized polyploid Brassica napus (Brassicaceae). Am J Bot 91: 174–183.

60. Henikoff S, Comai L (2003) Single-Nucleotide Mutations for Plant Functional Genomics. Plant Biotechnology. Teixeira da Silva JA, (ed), Global Science Books, Ltd., London, Vol I, pp. 476–482.

61. Murray M, Thompson W (1980) Rapid isolation of high molecular weight plant DNA. Nucleic acids res 8: 4321.