A Two-Locus Global DNA Barcode for Land Plants: The Coding rbcL Gene Complements the Non-Coding trnH-psbA Spacer Region

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**Background.** A useful DNA barcode requires sufficient sequence variation to distinguish between species and ease of application across a broad range of taxa. Discovery of a DNA barcode for land plants has been limited by intrinsically lower rates of sequence evolution in plant genomes than that observed in animals. This low rate has complicated the trade-off in finding a locus that is universal and readily sequenced and has sufficiently high sequence divergence at the species-level.

**Methodology/Principal Findings.** Here, a global plant DNA barcode system is evaluated by comparing universal application and degree of sequence divergence for nine putative barcode loci, including coding and non-coding regions, singly and in pairs across a phylogenetically diverse set of 48 genera (two species per genus). No single locus could discriminate among species in a pair in more than 79% of genera, whereas discrimination increased to nearly 88% when the non-coding trnH-psbA spacer was paired with one of three coding loci, including rbcL. In silico trials were conducted in which DNA sequences from GenBank were used to further evaluate the discriminatory power of a subset of these loci. These trials supported the earlier observation that trnH-psbA coupled with rbcL can correctly identify and discriminate among related species.

**Conclusions/Significance.** A combination of the non-coding trnH-psbA spacer region and a portion of the coding rbcL gene is recommended as a two-locus global land plant barcode that provides the necessary universality and species discrimination.
vergence of this region makes it an unlikely universal marker for species-level identification. Six plastid coding regions (accD, matK, ndhF, rpoB2, rpoC1, and ycf3) also have been recommended as putative plant barcodes [see http://www.rbgkew.org.uk/barcoding/index.html], but no comparisons of their effectiveness have been published. Finally, even though the plastid rbcL gene has been discounted as a species-level discriminator [14–15], some researchers have suggested that this region should be included as a standard for comparison to other markers or as a barcode candidate itself [16–17]. The advantages of this gene are that it is easily amplified and sequenced in most land plants and it is regarded as a benchmark locus in phylogenetic investigations by providing a reliable placement of a taxon into a plant family and/or genus. However, despite the promise of these regions as putative single-locus barcodes the overall lower levels of mutation rates in plants compared to animals [11] may necessitate a multi-locus barcode to maximally discriminate among plant species [7–8].

The objectives of the current study are two-fold: 1) to quantify universal application (PCR and sequencing) and sequence divergence among a phylogenetically diverse set of species pairs for nine putative barcode loci and 2) to determine which loci, if more than one locus is required, will maximize species identification when combined as a barcode.

RESULTS

The nine loci varied widely in the universality of their primers and levels of sequence divergence, and hence their potential use as barcodes (Table 1; Figures 1, 2). Only two loci, trnH-psbA and rbcL-a, exhibited high PCR success with standard primers by amplifying 95.8% (46 of 48 genera) and 92.7% (43 of 48 genera), respectively, of the test species (Figure 1). Three loci, ITS1, trnH-psbA, and rpoB2, had a mean sequence divergence value greater than two percent while the remaining loci ranged between 0.2% and 1.55% (Tables 1, 2, Figure 2). In the Wilcoxon Signed rank tests ITS1 exhibited a significantly higher degree of divergence (5.7%) than all other loci, followed by trnH-psbA (2.69%), which was significantly more divergent than rpoB2 (2.05%), rpoC1 (1.38%), and rbcL-a (1.29%). Due to the low PCR success of matK, and hence the small number of available comparisons, this locus was not shown to be significantly different than any of the other loci, except ITS1. The coding loci rpoB2, rpoC1, and rbcL-a exhibited statistically equal sequence divergence values for the data set (Table 2).

The proportion of genera in which species in a pair could be differentiated also varied widely among loci (Table 1; Figure 3). The trnH-psbA spacer and ITS1 showed a much higher level of differentiation (32.6% and 81.5%, respectively) than the other seven loci, none of which had a value higher than 70%. If universal application is incorporated and all genera are considered, then the overall proportion of genera in which species in a pair were differentiated dropped considerably in ITS1 (45.8%) while trnH-psbA maintained the highest resolution (79.1%) and rbcL-a the second highest (62.5%) with values for all other loci at 50% or less. Six genera were invariant between species in a pair for all of the candidate loci (Citrus, Eucalptus, Lycoris, Magnolia, Raphanus, and Sabal).

The results from data-mining sequences in GenBank, notwithstanding the drawbacks of using such data (e.g., unreliable identifications and uneven sequence quality [18]) and the relatively crude nature of the BLAST search engine, indicated that trnH-psbA was successful at returning a correct match. These tests using BLAST were employed as a complement to the primary results on barcode loci derived from the empirical comparative sequence data set. Many of the putative loci had too few sequences in GenBank to conduct a robust test (accD, ndhF, rpoB2, rpoC1, and ycf3) or were ruled out due to limitations in universal application (ITS1 and matK). For these reasons the in silico tests were not exhaustive and only focused on trnH-psbA and rbcL. Of the 103 genera tested, 75.7% (78 genera) of the searches identified the target sequence as the single best match with the BLASTn search. Similarly rbcL, which is a gene noted for its utility as a phylogenetic marker at the rank of family and genus, also demonstrated utility as a species-level identifier in the comparative data-mining tests [17]. Of the original 103 genera tested for trnH-psbA, 59 had rbcL sequences available in GenBank; of those 59 genera 76.3% (45 genera) of the searches identified the target sequence as the single best match with a BLASTn search (Table 3; Table S1). In the remaining 14 rbcL trials in which the correct species was not matched, the search returned more than one species in the correct genus (nine cases) or correct family (five cases). The repeated trials for the trnH-psbA spacer with this reduced data set resulted in a slightly higher percentage of success (83.0%) at identification at the species level; the remaining cases identified to the correct genus (Table 3; Table S1). The effect of number of sequences available for a genus in GenBank on the incidence of unique identifications was not statistically significant for either the trnH-psbA spacer (t = 1.49; df = 96; P = 0.14) or rbcL (t = 1.26; df = 57; p = 0.21). For the trnH-psbA spacer there was also no statistical difference between using partial sequences versus complete sequences in the searches: partial sequences resulted in 28.6% multiple matches while complete sequences resulted in 26.5% multiple matches (Chi-square 0.04; df = 1; p = 0.8).

The various combinations of two loci in the multi-locus tests were all more powerful at differentiating between species than either locus individually (Table 4). The trnH-psbA spacer when combined with either rbcL-a, rpoB2, or rpoC1 demonstrated the highest PCR primer success (100%, i.e., primers amplified for at least one if not both loci across all taxa) and the highest proportion of differentiated species pairs (87.5%; Table 4). The other two-locus combinations that exhibited a proportion of differentiated species pairs better than or equal to the best single locus were trnH-psbA+ITS1 (85.1%) and rbcL-a+matK (92.6%). The PCR success for these two combinations was 99% and 95.3%, respectively. The remaining combinations of loci showed differentiation of species in a pair in less than 82% of the genera.

The results of the GenBank two-locus data-mining tests of rbcL and trnH-psbA showed that together the two loci provided correct matches at the species level in 95.0% of the trials (Table 3). For the three cases in which the correct species was not matched in the BLASTn search, the query sequence was correctly identified to the appropriate genus.

The differences in the success of discrimination and sequence matching from combining the original sequence data (Table 4) and the BLASTn searches are primarily due to sample size and taxon selection. For the empirical tests (Table 4), taxonomically difficult taxa (e.g., palms, orchids, cycads) were intentionally selected in order to provide a robust test of how well the loci could resolve these species pairs. Whereas the result from the GenBank searches (Table 3) does not necessarily emphasize taxonomically difficult groups and instead reflects more closely the relative abundance of plant families. An increase in sampling of species in the empirical tests that reflects species diversity in nature (e.g., fewer palms and cycads and many more grasses and composites) would likely result in even higher success rates in discriminating between species pairs.
DISCUSSION

The results suggest that the non-coding *trnH-psbA* intergenic spacer remains the most viable candidate for a single-locus barcode for land plants [8]. In the expanded sampling of loci and taxa the *trnH-psbA* spacer continued to successfully address the trade-off between universal application and high sequence divergence. PCR priming sites within highly conserved flanking coding sequences combined with a non-coding region that exhibits high sequence divergence among species as well as diagnostic insertion/deletion mutations makes the *trnH-psbA* spacer highly suitable as a plant barcode. The significant length variation in *trnH-psbA* due to insertions, deletions, and simple sequence repeats as well as the genomic rearrangement of the inverted repeat in some monocots [19] could be considered as a possible limitation. Non-coding spacers can be difficult to align thereby limiting their utility in phylogenetic studies at higher taxonomic levels [20]. However, this issue has minimal effect on barcoding because the primary goal is species identification and not phylogenetic reconstruction that requires correct alignments. As demonstrated here for *trnH-psbA* GenBank BLASTn searches can find the correct match despite
sequence length variation and gaps and thus allow the presence of indels in a target barcode sequence. The local alignment algorithm currently used in a BLASTn search should be improved by substituting a global alignment algorithm, such as the one used in the Barcode of Life Data System (BOLD)[21], that is more efficient at aligning sequences with significant length variation and therefore more successful at matching them within a known sequence database. Search algorithms that use indels as characters should then have greater power to discriminate through exclusion of sequences that do not align and thereby reduce the database population against which the query sequence is compared [22].

The trnH-psbA spacer is the most promising single locus for a land plant barcode according to the criteria of universal application and high sequence divergence among species. The intent of the present study was to use these criteria to compare the trnH-psbA spacer with other suggested barcode loci across land plants. Several of the plastid genes (matK, rbcL, rpoB2, and rpoC1) as well as the nuclear ITS region exhibit some features that would make each a possible candidate for a plant barcode (Table 1). However, each of these loci also possesses one or more significant flaws that make it less suitable either due to low PCR amplification success, low levels of sequence divergence, limited utility in non-angiosperms, and/or absence in some land plant lineages. For example, rpoB2 had a high mean sequence divergence value (2.05%), but poor PCR success in non-angiosperms (failed in all tested gymnosperms, ferns and all but one moss); rpoC1 had better PCR success (83.3%) than rpoB2, but a lower mutation rate (1.38%). The locus matK, which has been shown to be quite variable in numerous phylogenetic studies [20,23], had the lowest amplification success (39.3%) of all loci tested in this study. Further development of primer designs for matK and the other loci may improve amplification success, but none of these genes have highly conserved sites near the most variable parts of the locus and hence it is not likely that sufficiently universal primers will be developed. Interestingly, rbcL-a in some cases proved better than other coding loci as a barcode. The mean percent sequence divergence for rbcL-a ranked sixth, but it exceeded all other loci except ITS1 and trnH-psbA in the percent of genera in which species pairs could be differentiated (69.8%). PCR success in rbcL-a was also very high (92.7%). ITS1, which was earlier suggested as a possible barcode

Figure 2. Properties of nine plant loci tested as putative barcodes. Blue bars indicate PCR success; yellow bars indicate percent success in differentiating between species of a pair; maroon bars indicate PCR success combined with the ability to differentiate between species of a pair. doi:10.1371/journal.pone.0000508.g002
Table 1. Comparison of results for nine individual loci tested as putative barcodes on 46–48 species pairs of land plants.

| Region                  | ITS1 | trnH-psbA | rbcL-a | matK | rpoC1 | ycf5 | rpoB2 | ndhJ | accD |
|-------------------------|------|-----------|--------|------|-------|------|-------|------|------|
| Species pairs tested    | 48   | 48        | 48     | 46   | 48    | 48   | 48    | 47   | 48   |
| Mean locus length (bp; standard deviation) | 300 (31.4) | 373 (147) | 530 (27.5) | 501 (18.4) | 531 (31.9) | 214 (16.8) | 485 (15.5) | 387 (4) | 293 (20.8) |
| Percent PCR success     | 60.4% | 95.8%     | 92.7%  | 39.3% | 83.3% | 50.0% | 77.1% | 69.1% | 78.1% |
| 2 species of pair       | 42   | 42        | 42     | 20   | 20    | 20   | 20    | 20   | 20   |
| 1 species of pair       | 2    | 2         | 2      | 1    | 1     | 1    | 1     | 1    | 1    |
| 0 species of pair       | 1    | 1         | 1      | 0    | 0     | 0    | 0     | 0    | 0    |
| Angiosperms (80 species)| 56   | 76        | 74     | 36   | 77    | 47   | 73    | 65   | 72   |
| Gymnosperms (4 species) | 0    | 0         | 0      | 0    | 0     | 0    | 0     | 0    | 0    |
| Ferns (4 species)       | 0    | 0         | 0      | 0    | 0     | 0    | 0     | 0    | 0    |
| Mosses (8 species)       | 2    | 8         | 8      | 0    | 3     | 0    | 1     | 0    | 1    |
| Mean percent sequence divergence (n; range; standard deviation)* | 5.7% (27; 14.4–0; 4.58) | 2.69% (43; 16.3–0; 3.54) | 1.29% (43; 10.1–0; 2.07) | 1.13% (14; 14.2–0; 3.76) | 1.38% (40; 18–0; 4.14) | 1.55% (21; 15.3–0; 3.51) | 2.05% (8; 15.0–0; 3.65) | 0.20% (28; 2.09–0; 0.527) | 1.2% (32; 13.9–0; 1.39) |
| Proportion of genera in which species were differentiated (n/n)** | 81.5% (22/27) | 82.6% (38/46) | 69.8% (30/43) | 64.3% (9/14) | 60% (24/40) | 61.9% (13/21) | 61.8% (21/34) | 44% (1/28) | 40.6% (13/32) |
| Total proportion of genera in which species were differentiated (n/n)*** | 45.8% (22/48) | 79.1% (34/48) | 62.5% (30/48) | 14.6% (9/64) | 50% (24/48) | 27.0% (13/48) | 43.8% (21/48) | 25.0% (11/44) | 27.2% (13/48) |

*Mean percent sequence divergence between species pairs across genera that were successfully amplified (n = # of species pairs)
**Proportion of genera in which both species were successfully amplified and exhibited sequence divergence between species (n/n = # of genera in which species of a pair were differentiated/total # of pairs amplified)
***Proportion of all genera regardless of successful amplification that exhibited sequence divergence between species (n/n = # of genera in which species of a pair were differentiated/total # of pairs sampled)

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the present study three-locus systems demonstrated little or no gain
use requiring less cost and effort with the desired results. In fact in
locus approach because it is simply the most expedient system to
efforts have focused on a two-locus rather than a three or more
needed for maximal species identification in land plants. Here
power greater than 80% is required, then two or more loci will
not differentiate more than 80% of plant species. If discriminatory
barcode, the results reported here suggest that a single locus may
rate of identification should be possible in the future.

Faulty sequencing techniques, a significantly increase in success
taxonomic misidentification, i.e., supposedly different species of
genera are examples of overall low rates of sequence divergence,
inter-specific sequence differentiation [24] further reducing its application as a barcode. Three of the tested
genes have been shown to be absent in some major groups of land
plants, i.e., accD absent in grasses, ndhF absent in pines, ycf3 absent
in bryophytes [25], thereby disqualifying them for consideration as widely applicable plant barcodes.

Six of the 48 genera in our sample (Citrus, Euphorbia, Ludisia, Magnolia, Raphanus, and Sahal) were invariant at each of the nine
loci in the species pairs tested. Some of these genera are members of families that are known to show low levels of interspecific
sequence divergence (e.g., Arecaceae [26], Cycadaceae [27]) and
were selected for this reason to be tested in this study. The possible
explanations for the lack of sequence variation are several:
exceptionally low rates of sequence evolution in these taxa,
taxonomic misidentification, and experimental error. If these six
genera are examples of overall low rates of sequence divergence,
then effective barcoding of such taxa will be difficult no matter
which locus is selected. If the lack of sequence variation is due
taxonomic misidentification, i.e., supposedly different species of
a pair are actually the same species, or experimental error, i.e.,
faulty sequencing techniques, a significantly increase in success
rate of identification should be possible in the future.

Despite the promise of trnH-psbA as a candidate for a land plant
barcode, the results reported here suggest that a single locus may
differentiate more than 80% of plant species. If discriminatory
power greater than 80% is required, then two or more loci will be
needed for maximal species identification in land plants. Here
efforts have focused on a two-locus rather than a three or more
locus approach because it is simply the most expedient system to
use requiring less cost and effort with the desired results. In fact in
the present study three-locus systems demonstrated little or no gain
over two-locus systems in the proportion of species in a pair that
could be differentiated.

A two-locus combinatorial method has been suggested previously [7–8,28], but has never been satisfactorily tested. The results of both generating new test sequences across land plants (Table 4) and in data mining GenBank (Table 3) demonstrate the
utility of this approach. The loci chosen should complement each
other both in terms of the lineages within which each can discriminate and in balancing type I (incorrect species assignment)
and type II (false rejecting proper assignment) errors. The combination of the non-coding trnH-psbA spacer with one of three
coding regions, rbcL, rpoB2, or rpoC1, promises the highest
universality and the greatest ability to differentiate species pairs in
our sample. Complementing a rapidly evolving locus such as the
trnH-psbA spacer with a more conservative locus (such as the
coding locus trnH-psbA) can minimize type I errors (such that sequences
are robustly assigned to the correct genus at least) and type II
errors (higher rates of sequence divergence can discriminate among closely allied species in highly speciose genera). Thus rbcL
with its proven ease of amplification with broadly applicable primers across land plants and its proven ability to identify taxa at the
level of genus and family make it the most appropriate choice for a two-locus barcode coupled with trnH-psbA.

The balance of within- and between-species sequence variation is an important aspect of barcode identification [1–2,29] and
should be taken into account in the development of a barcode for
any group of organisms. Multiple samples per species were not
included in the present study to ascertain the level of intraspecific
sequence variation for each locus. Such trials are now underway.
However, prior reports demonstrate that both rbcL [30] and trnH-
psbA [28] show significantly lower levels of genetic divergence
within species than between species.

In conclusion a two-locus barcode that combines a subunit of the coding locus rbcL(rbcL-a) with the non-coding trnH-psbA spacer

| Locus pairs | Relative ranks | N  | P-value | Result               |
|-------------|----------------|----|---------|----------------------|
| trnH-psbA  | rpoB2          | W+ = 198, W- = 55 | 22 | p < 0.0211 | trnH-psbA > rpoB2     |
| trnH-psbA  | rbcL-a         | W+ = 501, W- = 60 | 33 | p < 8.466e-05| trnH-psbA > rbcL-a    |
| trnH-psbA  | ITS1           | W+ = 193, W- = 17 | 20 | p < 0.0004 | trnH-psbA < ITS1      |
| trnH-psbA  | rpoC1          | W+ = 293, W- = 53 | 26 | p < 0.00296| trnH-psbA > rpoC1     |
| trnH-psbA  | matK           | W+ = 26, W- = 40  | 11 | p < 0.5771 | trnH-psbA = matK      |
| rbcL-a     | rpoB2          | W+ = 184.5, W- = 221.50 | 28 | p < 0.6819 | rbcL-a = rpoB2       |
| rbcL-a     | ITS1           | W+ = 0, W- = 210  | 20 | p < 1.91e-06| rbcL-a < ITS1        |
| rbcL-a     | rpoC1          | W+ = 221, W- = 214 | 29 | p < 0.9483 | rbcL-a = rpoC1       |
| rbcL-a     | matK           | W+ = 38, W- = 28   | 11 | p < 0.7002 | rbcL-a = matK        |
| rpoB2      | ITS1           | W+ = 5, W- = 185   | 19 | p < 3.815e-05| rpoB2 < ITS1        |
| rpoB2      | rpoC1          | W+ = 118, W- = 92  | 20 | p < 0.6477 | rpoB2 = rpoC1       |
| rpoB2      | matK           | W+ = 12, W- = 24   | 8  | p < 0.4609 | rpoB2 = matK        |
| rpoC1      | ITS1           | W+ = 0, W- = 171   | 18 | p < 7.63e-06| rpoC1 < ITS1        |
| rpoC1      | matK           | W+ = 3, W- = 25    | 7  | p < 0.0712 | rpoC1 = matK        |
| ITS1       | ITS2           | W+ = 75, W- = 16   | 13 | p < 0.03979| ITS1 > ITS2         |
| ITS1       | matK           | W+ = 54, W- = 1     | 10 | p < 0.003906| ITS1 > matK        |

\(N\) is the number of genera for which differences in divergence rate were compared, \(P\)-value is one sided probability of divergence rates being equal. \(P\)-values less than 0.05 were considered significant and interpreted to reflect significant differences in observed rates of divergence.

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is recommended. *rbcL*-a provides a strong recognition anchor that will place an unidentified specimen into a family, genus, and sometimes species; the highly variable *trnH-psbA* spacer will further narrow the correct species identification where *rbcL*-a lacks discriminating power, especially in species-rich genera of angiosperms. Both of these loci have standard primers currently available that make them universally amplifiable with the least effort in the broadest range of land plants. This two-locus plant barcode is now being applied to build a library of over 700 species of the world’s most important medicinal plants [31; Kress and Erickson, unpubl.]. This barcode library can then be used to test the identity and purity of plant-based medicines and herals, such as ginseng, ginkgo, echinacea, and St. John’s wort, sold in commercial markets and used by consumers. The results of this effort will contribute to the suite of uses of DNA barcodes with substantial economic and social value.

**MATERIALS AND METHODS**

**Tests of a single-locus barcode**

Pairs of species from 48 phylogenetically diverse plant genera (of 43 families in 39 orders; Figure 1; Table S2) were compared to quantify levels of interspecific sequence divergence at nine putative barcode loci. The set of taxa includes angiosperms, gymnosperms, ferns, mosses, and liverworts (40 of 48 genera were flowering plants; Figure 1). The selection of plant families and genera for each order was based on availability of tissue samples. The individual species within a genus were chosen without a priori expectation of relatedness, hence the congeneric pairs do not necessarily represent nearest neighbor species. Because the experiment was focused on comparing the discriminating power of loci the inclusion of at least some species pairs that could be resolved by all loci increases the statistical power to differentiate among loci. Only a single individual per species was included in the analysis (see comments on intraspecific variation in Discussion). Tissues (leaves for higher plants, thalli for mosses/liverwort) were collected fresh and dried in silica-gel, or recovered from preserved herbarium specimens of various ages; vouchers with institutional accession numbers were prepared for each sample and are stored at the United States National Herbarium at the Smithsonian Institution’s National Museum of Natural History. In addition some tissue samples were obtained from the United States Department of Agriculture germplasm resource network and are identified by a discrete USDA accession number (see Table S2).

Uniform DNA extractions were performed on tissue from all species using the DNeasy Plant Mini™ kit (Qiagen, CA). Dry plant material was disrupted in individual lysing tubes with a bead-mill. DNA extraction was conducted following manufacturer’s protocols. For all taxa and loci, we conducted PCR amplification in a two stage trial. The first stage used a standard (non-hot-start) DNA polymerase (Biolase™ Taq Polymerase, Bioline) in 25 ul reactions following the protocols of Kress, Wurdack, Zimmer,
Weigt and Janzen [8]. The second stage included only samples that did not amplify or that produced multiple PCR products. Samples of both types of failure were re-amplified using a hot-start DNA polymerase (Amplitaq-Gold™ DNA polymerase from Applied Biosystems, CA). The samples that failed to amplify were repeated at lower stringency, (50°C annealing temperatures, and 40 cycles), whereas samples that produced multiple PCR products were repeated at higher stringency (55°C annealing temperatures and 30 cycles). PCR products were then purified for sequencing with ExoSap-IT™ (USB Corp., Ohio) digestion (diluted 4:1 with water) and subsequently used as the template in a 12 μl sequencing reaction. Sequencing reactions were purified by gel

| Locus                  | Percentage of single matches to species-level (number of single matches; mean # of sequences/genus; standard deviation) | Percentage of single matches to genus/family-level (number of single matches; mean # of sequences/genus; standard deviation) |
|------------------------|-------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------|
| rbcl-a                 | 76.3% (45; 8.2; 13.6) | 23.7% (14; 12.8; 13.8) |
| trnH-psbA              | 83.0% (49; 19.1; 17.9) | 17.0% (10; 19.8; 12.1) |
| rbcl-a+trnH-psbA       | 95.0% (56; n/a; n/a) | 5.0% (3; n/a; n/a) |

59 genera, which had sequences available for both loci, were included in the test.

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Table 4. Comparisons of results for pairs of two loci for trnH-psbA, rpoB, rpoC, rbcl-a, matK, and ITS tested in all combinations as putative barcodes on 48 species pairs of land plants.

| Region                  | trnH-psbA-rbcl-a | trnH-psbA-rpoB2 | trnH-psbA+rpoC1 | trnH-psbA+ITS1 | rpoB2-rbcl-a | rpoB2+rbcl-a |
|-------------------------|------------------|-----------------|-----------------|---------------|--------------|--------------|
| Percent PCR success*    | 100% (96/96)     | 100% (96/96)    | 100% (96/96)    | 99% (95/96)   | 100% (48/48) | 90.6% (87/96) |
| 2 species               | 48               | 48              | 48              | 47            | 46           | 43           |
| 1 species               | 0                | 0               | 0               | 1             | 0            | 1            |
| 0 species               | 0                | 0               | 0               | 0             | 2            | 0            |
| Angiosperms (80)        | 80               | 80              | 80              | 79            | 76           | 80           |
| Gymnosperms (4)         | 4                | 4               | 4               | 4             | 4            | 0            |
| Ferns (4)               | 4                | 4               | 4               | 4             | 4            | 2            |
| Mosses (8)              | 8                | 8               | 8               | 8             | 8            | 8            |
| Proportion of genera in which species were differentiated (n/n)** | 87.5% (42/48) | 87.5% (42/48) | 87.5% (42/48) | 85.1% (40/47) | 78.3% (36/46) | 77.1% (37/48) | 70% (30/43) |
| Total proportion of genera in which species were differentiated (n/n)** | 87.5% (42/48) | 87.5% (42/48) | 87.5% (42/48) | 83.3% (40/48) | 75% (36/48) | 77.1% (37/48) | 62.5% (30/48) |
| Angiosperms only (n = 40) | 85% (34/40) | 85% (34/40) | 85% (34/40) | 82.5% (33/40) | 70% (28/40) | 72.5% (29/40) | 70% (28/40) |

| Region                  | rpoB2+ITS1 | rpoB2+matK | rpoC1+matK | rpoC1+ITS1 | rbcl-a+rpoB2 | rbcl-a+rbcl-a |
|-------------------------|------------|------------|------------|------------|--------------|--------------|
| Percent PCR success*    | 83.3% (80/96) | 83.3% (80/96) | 86.5% (83/96) | 89.6% (86/96) | 100% (96/96) | 100% (96/96) | 95.8% (92/96) | 70.8% (68/96) |
| 2 species               | 40          | 40         | 40         | 42         | 48           | 48           | 46           | 32          |
| 1 species               | 2           | 0          | 3          | 2          | 0            | 0            | 0            | 4           |
| 0 species               | 6           | 8          | 5          | 4          | 0            | 0            | 0            | 12          |
| Angiosperms (80)        | 78          | 80         | 78         | 80         | 80           | 80           | 76           | 66          |
| Gymnosperms (4)         | 0           | 0          | 0          | 0          | 4            | 4            | 4            | 0           |
| Ferns (4)               | 0           | 0          | 2          | 2          | 4            | 4            | 4            | 0           |
| Mosses (8)              | 2           | 0          | 3          | 4          | 8            | 8            | 8            | 2           |
| Proportion of genera in which species were differentiated (n/n)** | 80.0% (32/40) | 67.5% (27/40) | 70.0% (28/40) | 78.6% (33/42) | 77.1% (37/48) | 74.5% (35/47) | 82.6% (38/46) | 81.3% (26/32) |
| Total proportion of genera in which species were differentiated (n/n)** | 66.7% (32/48) | 56.3% (27/48) | 58.3% (28/48) | 68.8% (33/48) | 77.1% (37/48) | 72.3% (35/48) | 79.2% (38/48) | 54.2% (26/48) |
| Angiosperms only (40 pairs) | 80% (32/40) | 67.5% (27/40) | 65% (26/40) | 77.5% (31/40) | 72.5% (29/40) | 70% (28/40) | 75% (30/40) | 80% (32/40) |

*PCR amplification of either locus for members of a generic pair is regarded as successful amplification for that generic pair. **Proportion of genera in which both species were successfully amplified and exhibited sequence divergence between species (n/n= # of genera in which species of a pair were differentiated/total # of pairs amplified). ***Proportion of all genera regardless of successful amplification that exhibited sequence divergence between species (n/n= # of genera in which species of a pair were differentiated/total # of pairs sampled).
flitrations with Sephadex G-50 (Amersham Pharmacia Biotech), and then analyzed on an ABI3100 automated sequencer. DNA sequence trace files were aligned with the program Sequencher™ (Gene Codes Corp, MI), and analyzed for levels of sequence divergence as described below. For all loci, alignments between species of a pair were unambiguous and not problematic.

The potential of nine loci as barcodes were compared in this study. The term “locus” is not applied in the strict genetic sense and for convenience refers to both coding and non-coding regions in this discussion. Each of the putative barcodes derived from the seven coding loci represents a subset of the gene that exhibited the highest level of sequence variation and universal amplification within an easily sequenced read length (<700 bp). Six of the loci are described at http://www.rbgkew.org.uk/barcoding/index.html. A 550–600 bp subset of the rbcL molecule (termed rbcLa) located at the 5’ end of the large subunit that exhibited maximal sequence variation and universal amplification was also included in the analysis. All available combinations of primers for each of these seven loci were tested on a subset of 4 divergent taxa to select the primer sequences that were subsequently used throughout the experiment (Table S3).

Two spacer regions, one in the nuclear genome (ITS) and one in the plastid genome (trnH-psbA), were tested along with the coding loci. The two components of the nuclear internal transcribed spacer (ITS 1 and 2) were compared across 13 of the test genera for size and variability. The ITS1 subset produced a consistently higher level of sequence variation and universal amplification than ITS2. The potential of nine loci as barcodes were compared in this study. Six of the loci (trnH-psbA, ITS, rbcLa, ycf1, ycf2, in at least one important group of land plants (see Discussion).

In silico tests of single- and multi-locus barcodes
The sequencing trials of the 48 genera were complemented with data-mining experiments using sequences of candidate barcode loci from GenBank, which is the major repository for data supported by the United States National Center for Biotechnology Information. Although GenBank is not a substitute for a “barcode library,” which will need to be built with high quality DNA sequences from verified voucher specimens, the sequences currently available can provide an independent data set to test the discriminatory powers of various loci. Sufficient sequence records for two of the four most promising loci, trnH-psbA and rbcL, were available in GenBank whereas accessions for the other two coding loci, ycf1 and ycf2, were insufficient for meaningful comparisons. Sequences for a total of 103 genera (including angiosperms and gymnosperms, but no ferns or mosses) for which six or more full length or partial sequences for trnH-psbA were identified and recovered from GenBank. A species sequence representing each genus was used then used as a query sequence in a BLASTn search (short nearly exact search)[32], which is the core search engine available in GenBank for matching sequences. The search returned either a single match (i.e., where the query sequence was returned as the most likely match) or as multiple matches (i.e., where the query sequence plus one or more identical sequences were returned as equally likely). As a comparison, the same set of taxa tested for trnH-psbA was used to test the utility of rbcL as a complementary locus. Of the 103 genera used in the trnH-psbA trials, 59 had corresponding rbcL sequences available in GenBank. These 59 genera were queried in the same fashion as above for the portion of rbcL and as a repeat trial for the trnH-psbA spacer. These 59 genera were then used to test the success of a combined two-locus approach using the BLASTn search. T-tests (for paired samples [33]) were used to determine if the number of sequences available for a genus in GenBank would bias a BLASTn search towards returning a single match versus multiple matches.

SUPPORTING INFORMATION

Table S1 BLASTn trials on 59 genera with both trnH-psbA and rbcL sequences extracted from GenBank.

Table S2 Taxa sampled in tests of nine putative plant barcode loci.

Table S3 Primer sequences for test loci.

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Author Contributions
Conceived and designed the experiments: WK DE. Performed the experiments: DE. Analyzed the data: WK DE. Contributed reagents/materials/analysis tools: WK DE. Wrote the paper: WK DE.
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