RESEARCH ARTICLE

Novel Genetic Resources in the Genus Vigna Unveiled from Gene Bank Accessions

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Abstract

The genus Vigna (Fabaceae) consists of five subgenera, and includes more than 100 wild species. In Vigna, 10 crops have been domesticated from three subgenera, Vigna, Plectrotropis, and Ceratotropis. The habitats of wild Vigna species are so diverse that their genomes could harbor various genes responsible for environmental stress adaptation, which could lead to innovations in agriculture. Since some of the gene bank Vigna accessions were unidentified and they seemed to be novel genetic resources, these accessions were identified based on morphological traits. The phylogenetic positions were estimated based on the DNA sequences of nuclear rDNA-ITS and chloroplast atpB-rbcL spacer regions. Based on the results, the potential usefulness of the recently described species V. indica and V. sahyadriana, and some wild Vigna species, i.e., V. aconitifolia, V. dalzelliana, V. khandalensis, V. marina var. oblonga, and V. vexillata, was discussed.

Introduction

The genus Vigna, in the family Fabaceae, comprises more than 100 wild species [1]. It is an agriculturally important taxon, which includes 10 domesticated species (crops) such as cowpea (Vigna unguiculata (L.) Walpers), mung bean (Vigna radiata (L.) Wilczek) and azuki bean (Vigna angularis (Willd.) Ohwi & Ohashi). Since some of their wild relatives inhabit extreme environments such as arid land, sandy beaches, and limestone karsts [2], they are expected to harbor adaptive genes, which could be used for developing stress-resistant crops for agriculturally unsuitable lands. Moreover, since they have evolved a symbiotic relationship with root-nodulating bacteria, which is also adapted to these extreme environments and contributes toward nitrogen fixation, these legumes have a high potential to contribute toward low-input sustainable agriculture [3, 4].

To introduce useful traits of wild relatives to related crops, interspecific hybridization is the most efficient and reliable strategy. Sequence-based phylogenetic relationships among species play a fundamental role as indicators to predict interspecific cross-compatibility. To increase the genetic diversity of a wild Vigna collection for environmental stress screening, Vigna
accessions were introduced from several gene banks. Since some of the gene bank accessions were unidentified and seemed to be novel genetic resources that have not been analyzed at the molecular level, these accessions were identified based on morphological traits, and were included in the phylogenetic analysis.

Although Maréchal et al. [5] described seven subgenera in the genus Vigna, two of them, Macrorhynchus and Sigmoidoctropis, have been proposed to be distinct genera, i.e., Wajira and Sigmoidoctropis, respectively, based on morphological and molecular phylogenetic analyses [6, 7]. Among the five subgenera presently recognized (Ceratotropis, Haydonia, Lasiospron, Plecotropis, and Vigna), crop species have been developed only from three subgenera (Ceratotropis, Plecotropis, and Vigna). Therefore, we have focused on the species belonging to these subgenera in the present study.

The subgenus Ceratotropis, also known as the Asian Vigna, is agronomically the most important taxonomic group, from which seven crops have been domesticated, i.e., moth bean (Vigna aconitifolia (Jacq.) Maréchal), minni payaru (Vigna stipulacea Kuntze), mung bean, black gram (Vigna mungo (L.) Hepper), creole bean (Vigna reflexo-pilosa Hayata), rice bean (Vigna umbellata (Thunb.) Ohwi & Ohashi), and adzuki bean (Vigna angularis (Willd.) Ohwi & Ohashi). Tomooka et al. [8] described 21 species, which were divided into three sections: five species in section Aconitifolii, 12 species in section Angulares, and four species in section Ceratotropis. Tomooka & Maxted, 12 species in section Angulares. Tomooka & Maxted, and four species described in the subgenus Ceratotropis. Although four new species were recently described in the subgenus Ceratotropis, their molecular phylogenetic positions have not been studied. In the present study, two newly described species (V. indica and V. sahyadriana) and four wild species (wild V. aconitifolia (Jacq.) Maréchal, Vigna dalzelliana (Thunb.) E. Mey. As such, the taxonomic treatments of the species in the subgenus Ceratotropis, which had not been used in previous molecular phylogenetic studies, were analyzed.

Maréchal et al. [5] described seven species, consisting of two sections in the subgenus Plecotropis (four species in section Plecotropis and three species in section Pseudoliebrechtsia). The subgenus Plecotropis contains a lesser known but potentially important food legume called ‘tuber cowpea’ (Vigna vexillata (L.) A. Rich.) [13]. This fully domesticated form is still cultivated in Bali and Timor, Indonesia. Maréchal et al. [5] recognized six botanical varieties (var. vexillata, angustifolia, doichonema, macrosperma, pluriflora, and yunnanensis) in V. vexillata. Among these varieties, var. macrosperma was reported as a domesticated taxa but its origin was unknown. Later, considering some proposals for new treatments [14, 15], Maxted et al. [16] accepted seven taxonomic varieties in V. vexillata (var. vexillata, angustifolia, davyi, dolichonema, lobatifolia, macrosperma, and ovata). V. vexillata var. davyi and V. vexillata var. lobatifolia were treated as distinct species (Vigna davyi H. Bol., Vigna lobatifolia Baker) in the subgenus Plecotropis in Maréchal et al. [5] V. vexillata var. ovata was formerly treated as Strophostyles capensis (Thunb.) E. Mey. As such, the taxonomic treatments of the species in the subgenus Plecotropis are still considered immature, and phylogenetic discussions based on molecular sequence information are necessary. In the present study, accessions of V. vexillata var. vexillata, var. angustifolia, var. lobatifolia, var. macrosperma, var. ovata, and Bali domesticated accessions were analyzed.

In the subgenus Vigna, from which cowpea (Vigna unguiculata) and bambara groundnut (Vigna subterranea) have been domesticated, Maréchal et al. [5] described 36 species in six sections (two species in section Catia, two in Comosae, one in Liebrechtsia, two in Macrodontae, nine in Reticulatae, and 20 in Vigna). Cowpea is classified under Catia, and bambara groundnut is in the section Vigna. For Vigna, we are currently focusing on Vigna marina (Burm.) Merrill, which inhabits sandy beaches, as a genetic resource for salinity tolerance, and Vigna luteola (Jacq.) Bentham, which inhabits riversides, as a flood-tolerant genetic resource.
These two species are closely related, and Padulosi and Ng [19] described *V. marina* ssp. *oblonga* Padulosi as being distributed in coastal areas of West Africa. Sonnante et al. [20] confirmed the genetic independence of *V. luteola*, *V. marina* ssp. *marina*, and *V. marina* ssp. *oblonga* based on isozymes and RAPD. In addition, they showed that *V. marina* ssp. *oblonga* was more closely related to *V. luteola* than to *V. marina* ssp. *marina*. However, *V. marina* ssp. *oblonga* was not included in subsequent phylogenetic analysis based on DNA sequences, although Pasquet et al. [15] described *V. marina* ssp. *oblonga* as being a synonym of *V. luteola*.

We therefore performed a phylogenetic characterization of the aforementioned taxa. To our knowledge, a phylogenetic study using DNA sequences had not been conducted on these taxa based on the DNA sequences of the internal transcribed spacer region of the ribosomal DNA on the nuclear genome (hereafter rDNA-ITS), and the *atpB-rbcL* intergenic spacer on the chloroplast genome (hereafter *atpB-rbcL*).

**Materials and Methods**

**Plant materials**

Seventy-one accessions of the genus *Vigna*, consisting of 28 species and three subgenera (*Ceratotropis*, *Plectrotropis*, and *Vigna*) conserved at the National Institute of Agrobiological Sciences, Japan, were used (Table 1). Originally, nine accessions were either unidentified, or seemed to be misidentified, as shown by the bold texts in Table 1. For the morphological analysis and DNA extraction, all the accessions were planted in six 0.3-L plastic pots (one seed/pot), and a 5-L plastic pot (six seeds/pot), and kept in a greenhouse where the temperature was maintained above 20°C with 12 hours of day length. The morphology of each plant was evaluated. For *V. aconitifolia*, weight of a hundred grains, pod shattering, and water absorbency of the seed were evaluated as domesticated traits. When evaluating pod shattering, 20 pods were dried overnight in a circulating incubator at 40°C. Twenty seeds were submerged in a Petri dish at 25°C for two days, and the number of seeds that absorbed water was recorded. We used common bean (*Phaseolus vulgaris* cv. Taisho-kintoki) as an outgroup for molecular phylogenetic analysis.

**DNA Sequencing**

We sequenced the rDNA-ITS and *atpB-rbcL* of 72 accessions. DNA was extracted from young leaves using a modified CTAB method [21]. PCR primers were designed according to the previous study [22]; C2 (5′-TCCTTCGGTAATGTAGATGC-3′) and G1 (5′-GGAGGAGAAGTTGATACAGG-3′) for rDNA-ITS, and AT1 (5′-AGAACCAGTAGTGTAAGAT-3′) and RB (5′-ACACCAGCTTTGAAATCCAAC-3′) for *atpB-rbcL*. The PCR mixture, containing KOD-Plus-Neo one unit (TOYOBO), 1 x PCR Buffer supplied by the manufacturer, 200 μM dNTPs, 1.5 mM MgSO4, 10 ng of the DNA template, and 0.2 μM of each primer pair, was prepared in a total volume of 50 μL. The PCR cycle was as follows: 94°C for 2 min, 35 cycles of 98°C for 10 sec and 68°C for 1 min. The amplified PCR product was mixed with 2 μL of ExoSAP-IT, which had been diluted 20-fold, and incubated at 37°C for 30 min, and 80°C for 15 min. The sequencing reaction was conducted according to the protocol of BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The reactant was precipitated using ethanol, dried, and dissolved in 10 μL Hi-Di Formamid. The mixture was treated at 95°C for 5 min, and the DNA sequence was determined using a ABI PRISM 3130xl DNA Analyzer (Applied Biosystems). Sequencing was repeated until the depth of each base was greater than five, and the nucleotide sequence was determined according to majority rule in cases where a single nucleotide polymorphism was present. The accession numbers of the sequence information deposited in the DNA Data Bank of Japan (www.ddbj.nig.ac.jp/) are shown in Table 1.
Table 1. Plant materials used for phylogenetic analysis, and the sequence length and accession No. of rDNA-ITS and atpB-rbcL deposited at DDBJ.

| ID | Section | Species Name | Status | Origin | JP No. | Original ID and Species Identification | rDNA-ITS Sequence Length (bp) | rDNA-ITS DDBJ Accession No. | atpB-rbcL Sequence Length (bp) | atpB-rbcL DDBJ Accession No. |
|----|---------|-------------|--------|--------|--------|----------------------------------------|-----------------------------|-------------------------------|--------------------------------|---------------------------------|
| 1  | Aconitifoliae | V. aconitifolia | Domesticated | India | 245857 | TNAU GB 2009TN58 | 562 | LC082015 | 700 | LC082267 |
| 2  | Aconitifoliae | V. aconitifolia | Domesticated | India | 245897 | TNAU GB 2009TN79 | 562 | LC082017 | 699 | LC082269 |
| 3  | Aconitifoliae | V. aconitifolia | Domesticated | Pakistan | 104332 | NIAS GB 2752(5) | 562 | LC082018 | 699 | LC082270 |
| 4  | Aconitifoliae | V. aconitifolia | Wild | India | 245864 | TNAU GB 2009TN66, Vigna sp. | 562 | LC082012 | 699 | LC082264 |
| 5  | Aconitifoliae | V. aconitifolia | Wild | India | 245865 | TNAU GB 2009TN67, Vigna sp. | 562 | LC082013 | 700 | LC082265 |
| 6  | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205894 | NIAS GB 2000S-11 | 561 | LC082016 | 699 | LC082268 |
| 7  | Aconitifoliae | V. aridicola | Wild | India | 245864 | TNAU GB 2009TN79, Vigna sp. | 562 | LC082017 | 699 | LC082269 |
| 8  | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205896 | NIAS GB 2000S-12 | 561 | LC082018 | 699 | LC082270 |
| 9  | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205897 | NIAS GB 2000S-13 | 561 | LC082019 | 699 | LC082271 |
| 10 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205898 | NIAS GB 2000S-14 | 561 | LC082020 | 699 | LC082272 |
| 11 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205899 | NIAS GB 2000S-15 | 561 | LC082021 | 699 | LC082273 |
| 12 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205900 | NIAS GB 2000S-16 | 561 | LC082022 | 699 | LC082274 |
| 13 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205901 | NIAS GB 2000S-17 | 561 | LC082023 | 699 | LC082275 |
| 14 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205902 | NIAS GB 2000S-18 | 561 | LC082024 | 699 | LC082276 |
| 15 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205903 | NIAS GB 2000S-19 | 561 | LC082025 | 699 | LC082277 |
| 16 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205904 | NIAS GB 2000S-20 | 561 | LC082026 | 699 | LC082278 |
| 17 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205905 | NIAS GB 2000S-21 | 561 | LC082027 | 699 | LC082279 |
| 18 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205906 | NIAS GB 2000S-22 | 561 | LC082028 | 699 | LC082280 |
| 19 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205907 | NIAS GB 2000S-23 | 561 | LC082029 | 699 | LC082281 |
| 20 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205908 | NIAS GB 2000S-24 | 561 | LC082030 | 699 | LC082282 |
| 21 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205909 | NIAS GB 2000S-25 | 561 | LC082031 | 699 | LC082283 |
| 22 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205910 | NIAS GB 2000S-26 | 561 | LC082032 | 699 | LC082284 |
| 23 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205911 | NIAS GB 2000S-27 | 561 | LC082033 | 699 | LC082285 |
| 24 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205912 | NIAS GB 2000S-28 | 561 | LC082034 | 699 | LC082286 |
| 25 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205913 | NIAS GB 2000S-29 | 561 | LC082035 | 699 | LC082287 |
| 26 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205914 | NIAS GB 2000S-30 | 561 | LC082036 | 699 | LC082288 |
| 27 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205915 | NIAS GB 2000S-31 | 561 | LC082037 | 699 | LC082289 |
| 28 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205916 | NIAS GB 2000S-32 | 561 | LC082038 | 699 | LC082290 |
| 29 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205917 | NIAS GB 2000S-33 | 561 | LC082039 | 699 | LC082291 |
| 30 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205918 | NIAS GB 2000S-34 | 561 | LC082040 | 699 | LC082292 |
| 31 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205919 | NIAS GB 2000S-35 | 561 | LC082041 | 699 | LC082293 |
| 32 | Aconitifoliae | V. aridicola | Wild | Sri Lanka | 205920 | NIAS GB 2000S-36 | 561 | LC082042 | 699 | LC082294 |

(Continued)
| ID  | Section       | Species Name         | Status   | Origin          | JP No.     | Original Conservation Site | Original ID and Species Identification | rDNA-ITS Sequence Length (bp) | rDNA-ITS DDBJ Accession No. | atpB-rbcL Sequence Length (bp) | atpB-rbcL DDBJ Accession No. |
|-----|---------------|----------------------|----------|-----------------|------------|----------------------------|---------------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| 33  | Angulares     | V. nepalensis        | Wild     | Nepal           | 107881     | NIAS GB Nepalen            | 557                                   | LC081994                     | 689                           | LC082246                      |                               |
| 34  | Angulares     | V. reflexo-pilosa var. glabra | Domesticated | Philippines | 109684 | AVRDC GB V1160 | 557                                   | LC081996                     | 698                           | LC082238                      |                               |
| 35  | Angulares     | V. reflexo-pilosa var. reflexo-pilosa | Wild     | Malaysia        | 108967     | NIAS GB                  | 510–1                                 | LC081987                     | 698                           | LC082239                      |                               |
| 36  | Angulares     | V. rikiuensis        | Wild     | Japan           | 108810     | NIAS GB Y-4-1             | 556                                   | LC082001                     | 692                           | LC082253                      |                               |
| 37  | Angulares     | V. tenuicaulis       | Wild     | Myanmar         | 227438     | NIAS GB KYONKADON         | 557                                   | LC081991                     | 688                           | LC082243                      |                               |
| 38  | Angulares     | V. tenuicaulis       | Wild     | Thailand        | 109682     | NIAS GB CED91122-(8)     | 557                                   | LC081990                     | 688                           | LC082242                      |                               |
| 39  | Angulares     | V. trinervia         | Wild     | Malaysia        | 108840     | NIAS GB 503–4             | 561                                   | LC064352                     | 698                           | LC064362                      |                               |
| 40  | Angulares     | V. umbellata         | Domesticated | Japan         | 99485      | NIAS GB Menaga           | 557                                   | LC081982                     | 689                           | LC082234                      |                               |
| 41  | Angulares     | V. umbellata         | Wild     | Thailand        | 210639     | NIAS GB 99T-2             | 557                                   | LC064307                     | 689                           | LC064328                      |                               |
| 42  | Angulares     | V. umbellata         | Wild     | Thailand        | 109675     | NIAS GB (6)-1-1          | 557                                   | LC081981                     | 689                           | LC082233                      |                               |
| 43  | Angulares     | V. umbellata         | Wild     | Thailand        | 210644     | NIAS GB 99T-9            | 557                                   | LC064303                     | 689                           | LC064324                      |                               |
| 44  | Angulares     | V. umbellata         | Domesticated | Japan         | 107862     | NIAS GB CED91119-(1)     | 562                                   | LC064345                     | 694                           | LC064355                      |                               |
| 45  | Angulares     | V. mungo var. mungo | Domesticated | Thailand | 109668     | NIAS GB Subsomotod       | 562                                   | LC064346                     | 689                           | LC064356                      |                               |
| 46  | Angulares     | V. mungo var. silvestris | Wild     | India           | 107874     | NBPGR TC2211             | 562                                   | LC064347                     | 690                           | LC064357                      |                               |
| 47  | Angulares     | V. radiata var. radiata | Domesticated | Thailand | 110830     | NIAS GB CN60             | 595                                   | LC064348                     | 688                           | LC064358                      |                               |
| 48  | Angulares     | V. radiata var. sublobata | Wild     | Madagascar       | 107877     | AVRDC GB TC1966          | 587                                   | LC064349                     | 688                           | LC064359                      |                               |
| 49  | Angulares     | V. radiata var. sublobata | Wild     | Papua N.G.      | 226874     | NIAS GB 2005PNG08       | 597                                   | LC082004                     | 688                           | LC082256                      |                               |
| 50  | Angulares     | V. sahyadriana       | Wild     | India           | 235420     | Australian GB AusTRCF104896, Vigna sp. | 568                                   | LC082003                     | 689                           | LC082255                      |                               |
| 51  | Angulares     | V. vexillata         | Wild     | India           | 110836     | Belgian GB NI 1135, V. radiata var. setulosa | 564                                   | LC064353                     | 688                           | LC064363                      |                               |
| 52  | Angulares     | V. vexillata         | Wild     | India           | 245506     | TNAU GB 2008TN32, V. hainiana | 559                                   | LC064354                     | 688                           | LC064364                      |                               |
| 53  | Plectrotropis | V. vexillata        | Domesticated | Indonesia | 235863     | Belgian GB NI 1858       | 560                                   | LC082032                     | 683                           | LC082284                      |                               |
| 54  | Plectrotropis | V. vexillata        | Wild     | Brazil          | 202337     | USDA GB PI 406391       | 562                                   | LC082035                     | 684                           | LC082287                      |                               |
| 55  | Plectrotropis | V. vexillata        | Wild     | Papua N.G.      | 230747     | NIAS GB 2006PNG-37      | 563                                   | LC082037                     | 683                           | LC082289                      |                               |
| 56  | Plectrotropis | V. vexillata        | Wild     | Suriname        | 202334     | USDA GB PI 406383       | 563                                   | LC082036                     | 684                           | LC082288                      |                               |
| 57  | Plectrotropis | V. vexillata var. angustifolia | Wild     | Columbia        | 235869     | Belgian GB NI 936       | 563                                   | LC082038                     | 684                           | LC082290                      |                               |
| 58  | Plectrotropis | V. vexillata var. lobatifolia | Wild     | Namibia         | 235903     | Belgian GB NI 546       | 557                                   | LC082031                     | 686                           | LC082283                      |                               |
| 59  | Plectrotropis | V. vexillata var. macrosperrma | Domesticated | Sudan        | 235905     | Belgian GB NI 111       | 559                                   | LC082034                     | 684                           | LC082286                      |                               |

(Continued)
| ID | Section | Species Name | Status | Origin        | JP No. | Original Conservation Site | Original ID and Species Identification | rDNA-ITS Sequence Length (bp) | rDNA-ITS DDBJ Accession No. | atpB-rbcL Sequence Length (bp) | atpB-rbcL DDBJ Accession No. |
|----|---------|--------------|--------|---------------|--------|-----------------------------|------------------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| 60 | Plectrotropis | V. vexillata var. ovata | Wild   | South Africa  | 235908 | Belgian GB                 | NI 1869                                  | 562                           | LC082033                      | 684                           | LC082285                      |
| 61 | Plectrotropis | V. vexillata var. vexillata | Wild   | Congo         | 235912 | Belgian GB                 | NI 245                                   | 563                           | LC082039                      | 684                           | LC082291                      |
|    |          |              |        |               |        |                            |                                          |                               |                               |                               |                               |
|    | Subgenus | Vigna        |        |               |        |                            |                                          |                               |                               |                               |                               |
| 62 | Catiang  | V. unguiculata | Domesticated | Nigeria | 86801 | IITA GB               | IT 84S 2246                             | 581                           | LC082027                      | 686                           | LC082279                      |
| 63 | Catiang  | V. unguiculata | Domesticated | Sudan    | 86877 | IITA GB               | TVU 11979                                | 581                           | LC082026                      | 686                           | LC082278                      |
| 64 | Catiang  | V. unguiculata | Domesticated | Sudan    | 86879 | IITA GB               | TVU 11986                                | 581                           | LC082028                      | 686                           | LC082280                      |
| 65 | Catiang  | V. unguiculata ssp. dekindtiana | Wild | Mali         | 89083 | IITA GB               | TVNU 457                                 | 575                           | LC082030                      | 684                           | LC082282                      |
| 66 | Catiang  | V. unguiculata ssp. sesquipedalis | Domesticated | Sri Lanka | 81610 | NIAS GB               | MA                                       | 581                           | LC082029                      | 686                           | LC082281                      |
| 67 | Vigna    | V. luteola    | Wild   | Australia     | 236246 | Australian GB           | AUSTRCF 320527                           | 566                           | LC082021                      | 689                           | LC082273                      |
| 68 | Vigna    | V. luteola    | Wild   | Brazil        | 235855 | Belgian GB             | NI 858                                   | 566                           | LC082023                      | 689                           | LC082275                      |
| 69 | Vigna    | V. marina ssp. marina | Wild | Japan      | 235813 | NIAS GB               | 2009RIO-1                                | 569                           | LC082022                      | 690                           | LC082274                      |
| 70 | Vigna    | V. marina ssp. oblonga | Wild | Benin       | 233389 | NIAS GB               | 2006BENIN29                              | 567                           | LC082024                      | 690                           | LC082276                      |
| 71 | Vigna    | V. subterranea | Domesticated | unknown | 79992 | NIAS GB               | L15-20-2                                | 575                           | LC082025                      | 690                           | LC082277                      |
| 72 | -        | Phaseolus vulgaris | Domesticated | Japan       | 219310 | NIAS GB               | TAISHOU KINTOKI                         | 554                           | LC082303                      | 679                           | LC082302                      |

Nine accessions which were originally either unidentified, or seemed to be misidentified are shown by bold texts.

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Multiple alignment was conducted for each rDNA-ITS and atpB-rbcL using Clustal W [23]. The sequence frame was determined according to the previous study [22], and the trimmed sequence was used to construct a phylogenetic tree by the maximum likelihood estimation using MEGA6 [24]. Bootstrap analysis was conducted with 1000 replications.

Results

Morphology-based species identification

Among the nine unidentified or misidentified accessions, six accessions were identified as the following four species (V. *aconitifolia*, V. *dalzelliana*, V. *indica*, and V. *sahyadriana*) based on morphological observation.

Accessions ID-4, ID-5, and ID-6, which were collected in India, were identified as the wild forms of moth bean (V. *aconitifolia*). Seedling, stipule, and seed morphologies of the domesticated and newly identified wild forms of V. *aconitifolia* are shown in Fig 1. Both domesticated and wild forms showed similar variations in leaflet shape, ranging from entire to deeply lobed. Only seeds of the wild forms were covered with a semi-transparent seed coat covering. While the domesticated forms were characterized by larger seeds with water-permeable seed coat and non-shattering pods, the wild forms were found to have smaller seeds, with a water-proof seed coat and high shattering pods (Table 2).

Morphologies of the seedling, style beak, and seed of the remaining accessions newly identified as V. *dalzelliana*, V. *indica*, and V. *sahyadriana* are shown in Fig 2. Accession ID-23, collected in southern Myanmar, showed hypogeal germination with petiolate primary leaves, glabrous pods, seeds without seed coat coverings (smooth seed coat), small yellow flowers, left curved keel petal with protuberance on left keel (keel pocket), indicating that this accession belonged to the section Angulares in the subgenus Ceratotropis. Additionally, it had a flat style beak (spoon-like shape), which is a key characteristic of V. *dalzelliana*. Therefore, we have identified this accession as V. *dalzelliana*.

Accession ID-50, collected in India, was introduced from the gene bank of Australia (AusTRCF104896), where it was treated as Vigna sp. (Table 1). It showed epigeal germination with sessile primary leaves, seeds with seed coat covering, hairy pods, yellow flower, and left curved keel petal with prominent protuberance on left keel petal (keel pocket), indicating that this accession belongs to the section Ceratotropis in the subgenus Ceratotropis. Seed morphology and very long style beak matched the characteristics of V. *mungo*, whereas the direction of laterally attaching pods to the peduncle matched that of V. *radiata*. These characteristics matched the key characters of V. *sahyadriana* well, which was described as a new species by Aitawade et al. [10].

Accession ID-10, collected in India, was introduced from the ILRI (International Livestock Research Institute) gene bank (IL-25019), where it was conserved as V. *trilobata*. It showed epigeal germination with sessile primary leaves, seeds with seed coat covering, hairy pods, small yellow flowers, left curved keel petal with a small protuberance on left keel petal (keel pocket), and a protruding growth habit with deeply lobed leaflets, indicating this accession belongs to the section Aconitifoliae in the subgenus Ceratotropis. At a glance, it had a very similar overall morphology to V. *trilobata*. However, its stipule was lanceolate, and its seed was rectangular with a very short, non-protruding hilum, which did not match the key characters of V. *trilobata*. These characteristics matched those of V. *indica*, which was described as a new species by Dixit et al. [9].

Accession ID-43, collected in Thailand, was originally identified as V. *umbellata*. However, it showed some features that did not match the key characteristics of V. *umbellata*. Accession ID-51, collected in northern India, was introduced from a Belgian gene bank (NI 1135) as V. *
| ID | Seedling | Stipule | Seed |
|----|----------|---------|------|
| 1  | ![Domesticated form](image1) | ![Stipule](image2) | ![Seed](image3) |
| 2  | ![Domesticated form](image1) | ![Stipule](image2) | ![Seed](image3) |
| 3  | ![Domesticated form](image1) | ![Stipule](image2) | ![Seed](image3) |
| 4  | ![Domesticated form](image1) | ![Stipule](image2) | ![Seed](image3) |
| 5  | ![Wild form](image1) | ![Stipule](image2) | ![Seed](image3) |
| 6  | ![Wild form](image1) | ![Stipule](image2) | ![Seed](image3) |

Fig 1. Domesticated form and wild ancestral form of moth bean (*V. aconitifolia*). Scale bars are 1 mm.
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radiata var. setulosa. Accession ID-52, collected in southern India, was introduced from the Tamil Nadu Agricultural University (TN32) as V. hainiana. Both of these accessions had a similar morphology to that of V. radiata in general. However, they showed some features that did not match the key characteristics of V. radiata. Therefore, we could not determine the taxonomic identification for these three accessions based on the morphological analysis in the present study.

Molecular phylogenetic analysis

DNA sequences of rDNA-ITS and atpB-rbcL were determined for 71 accessions of the genus Vigna. For rDNA-ITS, the total length ranged from 556–597 bp; V. minima, V. riukiuensis, and V. nakashimae had the shortest (556 bp), and V. radiata had the longest rDNA-ITS (587–597 bp). The total lengths of atpB-rbcL ranged from 683 to 700 bp; V. unguiculata and V. vexillata had the shortest (683–686 bp), whereas V. aconitifolia had the longest atpB-rbcL (699–700 bp) (Table 1). The numbers of polymorphic sites in rDNA-ITS and atpB-rbcL were 211 and 80, respectively.

Based on these sequences of rDNA-ITS and atpB-rbcL, phylogenetic trees for respective regions were constructed (Figs 3 and 4). In both phylogenetic trees, the subgenus Ceratotropis

| ID | Status   | Seed weight ± SD (g/100 grains) | Shattering pods (%) | Germination (%) |
|----|----------|---------------------------------|---------------------|-----------------|
| 1  | Domesticated | 3.39 ± 0.42 a                  | 0                   | 100             |
| 2  | Domesticated | 2.03 ± 0.38 b                  | 0                   | 100             |
| 3  | Domesticated | 2.20 ± 0.11 b                  | 0                   | 100             |
| 4  | Wild      | 0.86 ± 0.14 c                  | 100                 | 0               |
| 5  | Wild      | 1.15 ± 0.08 c                  | 100                 | 0               |
| 6  | Wild      | 1.26 ± 0.11 c                  | 100                 | 0               |

1 Averages of 3 replications. Different letters indicate that seed weights are significantly different, by Tukey—Kramer’s HSD test (P < 0.05).

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![Fig 2. Morphological characteristics of V. dalzelliana, V. sahyadriana, and V. indica. Scale bars are 0.3 mm with style beak, and 1 mm with seeds.](doi:10.1371/journal.pone.0147568.g002)
Fig 3. Maximum likelihood tree based on nuclear rDNA-ITS region for the genus *Vigna*, with *Phaseolus vulgaris* as an outgroup. Numbers beside branches represent bootstrap values (%) based on 1000 replications. Scale indicates genetic distance. Domesticated accessions are indicated with black circles, accessions which have been introduced as unidentified or misidentified accessions are indicated with red text, and taxa in which phylogenetic discussion using DNA sequences had not been conducted are indicated with blue text.

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Fig 4. Maximum likelihood tree based on chloroplast atpB-rbcL spacer region for the genus Vigna, with Phaseolus vulgaris as an outgroup.

Numbers beside branches represent bootstrap values (%) based on 1000 replications. Scale indicates genetic distance. Domesticated accessions are indicated with black circles, accessions which have been introduced as unidentified or misidentified accessions are indicated with red text, and taxa of which phylogenetic discussion using DNA sequences had not been conducted are indicated with blue text.

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formed a single cluster, distinct from the subgenera Vigna and Plectrotropis. The section Catiang in the subgenus Vigna allied with the subgenus Plectrotropis forming a single cluster, while the section Vigna in the subgenus Vigna was distantly allied.

The phylogenetic tree based on rDNA-ITS divided the section Aconitifoliae into multiple branches, and clustered the section Ceratotropis and Angulares independently (Fig 3). Alternatively, the phylogenetic tree based on atpB-rbcL divided the subgenus Ceratotropis into two groups, i.e., a blended group comprising the sections Aconitifoliae and Ceratotropis, and the section Angulares (Fig 4). While the section Angulares clustered distinctly from other groups, the interspecific genetic distances within the Angulares cluster were small.

The phylogenetic analysis revealed the species most closely related to the accessions that were newly identified in this study. Accession ID-4, ID-5, and ID-6, identified as a wild form of moth bean, were most closely related to moth bean (V. aconitifolia). Accession ID-23 (Myanmar), identified as V. dalzelliana, was most closely related to the V. dalzelliana collected in India. Accession ID-50, identified as V. sahyadriana, was most closely related to V. mungo. Accession ID-10, identified as V. indica, was most closely related to V. aconitifolia in the phylogenetic tree based on rDNA-ITS, and to V. subramanian in the phylogenetic tree based on atpB-rbcL. Accession ID-43 (Vigna sp.) was closely related to V. exilis in the rDNA-ITS tree, whereas it was allied with V. umbellata in the atpB-rbcL tree. Accessions ID-51 and ID-52 were most closely related to V. radiata in both trees.

V. khandalensis (accession ID-11) was differentiated substantially from other species, but was relatively close to V. stipulacea. Accessions within V. vexillata showed considerable levels of genetic variation. The accession ID-58 (V. vexillata var. lobatifolia), and the Indonesian domesticated form (accession ID-53) noticeably differentiated from other V. vexillata accessions. V. marina ssp. oblonga (accession ID-70), which was found on the coast of West Africa, was more closely related to V. luteola than to V. marina ssp. marina.

Discussion

Genetic differentiation within the genus Vigna

The subgenus Ceratotropis is thought to have emerged from the subgenus Vigna via the subgenus Plectrotropis [16, 25, 26]. The theoretical basis of this hypothesis is that, while the subgenus Vigna has a symmetric keel without pocket, the subgenus Plectrotropis has a curved keel with a pocket, and the subgenus Ceratotropis has a more prominently twisted keel with a more prolonged pocket. However, the phylogenetic tree using rDNA-ITS in this study suggested the following genetic differentiation patterns. The common ancestor of the genus Vigna first diverged into the common ancestor of the subgenera Vigna plus Plectrotropis, and the common ancestor of the subgenus Ceratotropis. Then, the common ancestor of the subgenera Vigna plus Plectrotropis diverged into the common ancestor of the section Vigna (subgenus Vigna) and the common ancestor of the section Catiang (subgenus Vigna) plus subgenus Plectrotropis. This is supported by the fact that the species in the section Catiang (subgenus Vigna) and the subgenus Plectrotropis have purple flowers, while those in the section Vigna (subgenus Vigna) have yellow flowers. Similar species relationships to our phylogenetic tree were obtained in previous studies using other molecular markers [7, 20, 27]. Therefore, it seems more appropriate to raise the rank of the section Catiang as a subgenus level. However, we leave this taxonomic revision for future work, since we used the limited number of species in the section Catiang, Vigna, and the subgenus Plectrotropis.

“Plectrotropis”, which represents the subgenus, and the section including V. vexillata, has been misspelled as “Plectotropis” in Maréchal et al. [5], and in many subsequent publications such as Tomooka et al. [8] and Maxted et al. [16], but the former should be the correct spelling,
as it appeared in Schumach [28] and Baker [29] as a genus name and a subgenus name, respectively.

After cowpea and *V. vexillata* were shown to be relatively close to each other by molecular analysis [30], an interspecific hybrid between the two species was obtained [31]. Moreover, an interspecific hybrid was obtained between cowpea and *V. luteola*, which are more distantly related species [32]. In the present study, we propose that *V. marina* is worth trying for producing interspecific hybrids with bambara groundnut (*V. subterranea*), based on their relatively close phylogenetic positions. *V. marina* is highly tolerant to salinity and alkaline soil [17, 33], while bambara groundnut is a crop that is adapted to arid lands [34]. Drought, saline, and alkaline soils are the most important environmental stresses to be addressed in agriculture.

**Novel Genetic Resources in the Genus *Vigna***

*Vigna indica* T.M. Dixit, K.V. Bhat & S.R. Yadav. Accession ID-10 is revealed to be the only germplasm of *V. indica* currently available at the gene bank. Although a holotype (*Rothe 6229a*) of this species was described as *V. trilobata* (L.) Verdcourt var. *pusilla* Naik et Pokle [35], results of the phylogenetic analysis supported Dixit et al. [9], in that this taxon is an independent species in the section *Aconitifoliae*. Whereas *V. indica* was reported to be morphologically most similar to *V. aridicola* by Dixit et al. [9], it was also similar to the wild form of *V. aconitifolia* in its stipule and flower morphology.

In this study, *V. indica* showed the closest relationship with *V. aconitifolia* in the rDNA-ITS tree. Conversely, it showed almost the same *atpB-rbcL* sequence as that of *V. subramaniana*. These facts suggest the possibility that *V. indica* is derived from an interspecific hybrid between *V. subramaniana* and *V. aconitifolia*. Further studies are necessary to confirm the origin of this species. Additionally, useful traits screening and interspecific cross-compatibility of *V. indica* should be conducted to determine its usefulness as a genetic resource, especially for moth bean (*V. aconitifolia*), the most closely related crop.

*Vigna sahyadriana* Aitawade, K.V. Bhat et S.R. Yadav. Accession ID-50 is the only germplasm of *V. sahyadriana* available from the gene bank at present. This species was recently described as a new species distributed in Maharashtra, India [10]. Since accession ID-50 was collected in Madhya Pradesh, India, the distribution range of this species seems to have expanded toward the inland of India.

Accession ID-50 was most closely related to, but clearly distinguishable from, black gram (*V. mungo*) in both phylogenetic trees (Figs 3 and 4). This suggests that the useful traits and interspecific cross-compatibility of *V. sahyadriana* should be investigated to determine if it can be used as genetic resources for black gram.

*Vigna aconitifolia* (Jacq.) Maréchal: Wild ancestor of moth bean. Although the wild form of moth bean was documented to be distributed in India [36], living samples have not been identified in the gene bank [27], and therefore its identity and useful traits have not been studied. In this study, we found the wild ancestor of moth bean in a gene bank collection. Accessions ID-5 and ID-6 were collected in Tamil Nadu, and accession ID-4 was collected in Andhra Pradesh, India. The collection sites of these three accessions suggest that the primary habitat of the wild form of moth bean is southeastern India.

Moth beans have been cultivated mainly in arid lands from India to Pakistan, and also in some other countries including Bangladesh, Myanmar, and China [37]. Since moth bean is reported as a crop most tolerant to drought and heat in the subgenus *Ceratotropis* [38, 39], it is generally thought to be suitable as a crop in tropical arid lands.

Recently, we have found that the wild ancestor of moth bean showed higher drought tolerance than the domesticated forms, and we successfully obtained the *F*₂ lines among the two
forms (data not shown). Moreover, since the interspecific hybrid between mung bean and moth bean has been reported [40], wild moth bean would be useful to develop moth bean and mung bean varieties with higher drought tolerance.

**Vigna dalzelliana** (O. Kuntze Verdcourt. The geographical distribution of this species was thought to be limited to India and Sri Lanka [8]. Although Thuan [41] reported *V. dalzelliana* in the Indo-China region (Vietnam, Laos, and Cambodia), it was the result of a misidentification of *V. minima* specimens [39]. More recently, John et al. [42] reported that they found *V. dalzelliana* in the Andaman Islands. Identification of accession ID-23 as *V. dalzelliana* in this study revealed an additional range of geographical distribution for this species, southern Myanmar.

The dissemination pathway of *V. dalzelliana* from India to southern Myanmar is unknown. Further explorations in the broad areas along the Bengal Gulf (Bangladesh and Myanmar) are necessary. However, since *V. dalzelliana* also inhabits Sri Lanka and the Andaman Islands [8, 42], researchers must consider the possibility that the distribution range expanded from India to Myanmar via these Islands.

Based on the rDNA-ITS tree, *V. dalzelliana* is located at the basal position with a *V. minima* species complex (*V. minima, V. nakashima, V. riukiensis*) [43], and both of these species are well differentiated within the section **Angulares** (Fig 3). Since *V. dalzelliana* is the only species known to be distributed in south India, where species of the other two sections are rich, it could be the ancestral species of the section **Angulares**. Investigating the process of the species emergence and expansion will provide important insights to understand the evolution of this section.

**Vigna khandalensis** (Santapau) Raghavan & Wadhwa. *Vigna khandalensis* was reported to inhabit a rainforest climate area in the Western Ghats and the Deccan Plateau in India [44]. It is the only wild species to have an erect plant type in the subgenus **Ceratotropis** in *Vigna*. Its seeds were collected as a food during famines [45]. While Tomooka et al. [8] classified this species in the section **Aconitifoliae** based on the short keel pocket and style beak; Bisht et al. [46] reported that this species is morphologically similar to species in the section **Ceratotropis**. The phylogenetic trees in this study suggested that *V. khandalensis* is a species in the section **Aconitifoliae**, and located at the basal position to the species in the section **Ceratotropis**. *V. khandalensis* was most closely related to *V. stipulacea* in the section **Aconitifoliae**, and the two species were similar in that they had large stipules. Since *V. stipulacea* is a creeping plant cultivated as food, fodder, and green manure in Tamil Nadu, India [2], *V. khandalensis* might be used to improve *V. stipulacea* growth. *V. khandalensis* may also be useful as a genetic resource for other section **Ceratotropis** crops, since the interspecific hybrid between this species and mung bean was obtained [47].

**Vigna marina** (Burm.) Merrill ssp. *oblonga* Padulosi. *V. marina ssp. oblonga* was proposed for the plants growing on the coastal zones of West Africa [19]. The phylogenetic tree using rDNA-ITS in this study confirmed that *V. marina ssp. oblonga* was more closely related to *V. luteola* than to *V. marina ssp. marina* (Fig 3), which was suggested by isozyme and RAPD analyses [20]. Additionally, phylogenetic trees suggest that there is a large intraspecific variation in *V. luteola*.

To address the evolution of *V. luteola* and *V. marina*, we need to consider *V. oblongifolia* A. Rich., a species closely related to these, although it was not included in this study. In *V. oblongifolia*, two botanical varieties have been described [25]. Phylogenetic trees in the previous studies have shown that *V. oblongifolia var. parviflora* is more closely related to *V. luteola* than to *V. marina*, and *V. oblongifolia var. oblongifolia* is more distant from these [48, 49]. This suggests that *V. marina ssp. oblonga* may be more closely related to *V. oblongifolia var. parviflora* than to *V. marina ssp. marina*. Therefore, the taxonomic treatment of *V. marina ssp. oblonga*,
and *V. oblongifolia* var. *parviflora* should be reconsidered based on intra and inter-specific variations in *V. marina*, *V. luteola*, and *V. oblongifolia*.

Since there are no interspecific crossing barriers among *V. marina* ssp. *marina*, *V. marina* ssp. *oblonga*, and *V. luteola* [17, 50], and interspecific hybrid plants between *V. oblongifolia* and *V. luteola* were obtained [51], these are thought to form a primary gene pool. Therefore, to introduce the salinity and alkaline tolerance of *V. marina* into bambara groundnut, interspecific cross-compatibility should be investigated, taking into consideration the use of bridging species in the section *Vigna*. In Maxted et al. [16], there are 18 species listed in the section *Vigna*.

**Vigna vexillata** (L.) A. Rich. The wild forms of this species are widely distributed in pantropical regions, including Africa, Asia, Oceania, and America, and its swollen roots have been collected as food [52–54]. This species includes two domesticated forms that are morphologically distinct from each other. One is a twining plant without any taxonomic rank at an intraspecific level, which is cultivated in Bali, Indonesia [13]. Another is an erect plant named *V. vexillata* var. *macropserma*, which is collected in Africa, Central America, and Australia. For both, the domestication origins are unknown.

In this study, the Indonesian domesticated form (accession ID-53) was found to be genetically differentiated from other species. This suggests that the Indonesian domesticated form, and *V. vexillata* var. *macropserma* (accession ID-59), have been domesticated independently from different wild forms. This notion was also supported by the fact that a hybrid among the two domesticated forms was not obtained [55]. Moreover, there is an intraspecific crossing barrier between the Indonesian domesticated form and some wild forms [55]. Therefore, the ancestor of the Indonesian domesticated form is unknown.

Similarly, *V. vexillata* var. *lobatifolia* was found to be genetically differentiated from other species. This taxon was described originally as *V. lobatifolia* Baker [56], then classified as an independent species in the section *Pseudoliebrechtsia* [25], or the section *Plectrotropis* [5] in the subgenus *Plectrotropis*, and then given the current rank as botanical variety of *V. vexillata* based on isozyme polymorphisms [15, 16, 57]. However, since *lobatifolia* has a unique habitat (Namib Desert), and is morphologically distinct, we do not reject the taxonomic systems of Verdcourt [25] and Maréchal et al. [5], in which it was treated as an independent species. However, only nine accessions in five varieties of *V. vexillata* were analyzed for the subgenus *Plectrotropis* in this study, and thus further studies are required to systematize the taxonomy of this subgenus, and clarify the rank of the Indonesian domesticated forms and *V. vexillata* var. *lobatifolia*.

The natural habitat of *V. vexillata* was very diverse, including arid lands, coastal areas, acidic soil, and alkaline soil [16, 58, 59]. Some accessions have been reported to harbor flood resistance and pest resistance [60–63]. It is therefore believed that this species contains highly useful genetic resources to breed crops for agriculturally unfavorable lands.

**Future perspectives**

In recent years, research on the use of wild relatives has been actively pursued. In addition to interspecific cross-breeding, new concepts have been proposed such as ‘Reverse Breeding’ [64], which involves regaining the crop stress tolerance, which has been lost in the breeding or domestication process, by backcrossing with the wild form. Another strategy is ‘Neo-Domestication’ [18], or the domestication of the stress-tolerant wild species that cannot be crossed with crop species. This process could be achieved by using mutation breeding, and mutant screening could be accelerated by TILLING, a screening method using the sequence information of domesticated genes. To advance these wild species breeding strategies, more information...
concerning the correct taxonomic placement, and genetic relationships among species, should be acquired to predict interspecific cross-compatibility, and to select an appropriate breeding strategy.

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Author Contributions

Conceived and designed the experiments: YT PS NT. Performed the experiments: YT CM KI KN. Analyzed the data: YT CM. Contributed reagents/materials/analysis tools: YT PS MP NS NT. Wrote the paper: YT NT.

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