Geographical origin of *Leucobryum boninense* Sull. & Lesq. (Leucobryaceae, Musci) endemic to the Bonin Islands, Japan

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Abstract

*Leucobryum boninense* is endemic to the Bonin Islands, Japan, and its related species are widely distributed in Asia and the Pacific. We aimed to clarify the phylogenetic relationships among *Leucobryum* species and infer the origin of *L. boninense*. We also describe the utility of the chloroplast *trnK* intron including *matK* for resolving the phylogenetic relationships among *Leucobryum* species, as phylogenetic analyses using *trnK* intron and/or *matK* have not been performed well in bryophytes to date. Fifty samples containing 15 species of *Leucobryum* from Asia and the Pacific were examined for six chloroplast DNA regions including *rbcL*, *rps4*, partial 5′ *trnK* intron, *matK*, partial 3′ *trnK* intron, and *trnL*-F intergenic spacer plus one nuclear DNA region including ITS. A molecular phylogenetic tree showed that *L. boninense* made a clade with *L. scabrum* from Japan, Taiwan and, Hong Kong; *L. javense* which is widely distributed in East and Southeast Asia, and *L. pachyphyllum* and *L. seemannii* restricted to the Hawaii Islands, as well as with *L. scaberulum* from the Ryukyus, Japan, Taiwan, and southeastern China. *Leucobryum boninense* from various islands of the Bonin Islands made a monophylic group that was closely related to *L. scabrum* and *L. javense* from Japan. Therefore, *L. boninense* may have evolved from *L. scabrum* from Japan, Taiwan, or Hong Kong, or *L. javense* from Japan. We also described the utility of *trnK* intron including *matK*. A percentage of the parsimony-informative characters in *trnK* intron sequence data (5.8%) was significantly higher than that from other chloroplast regions, *rbcL* (2.4%) and *rps4* (3.2%) sequence data. Nucleotide sequence data of the *trnK* intron including *matK* are more informative than other chloroplast DNA regions for identifying the phylogenetic relationships among *Leucobryum* species.

Introduction

Bryophyte species tend to have broad geographical distribution with a morphological uniformity in comparison with those of seed plants. In the Northern Hemisphere, more than 60% of the flora of the Arctic and boreal regions is made up of the same species (Schofield and Crum 1972). A single sporangium of a bryophyte may contain thousands and sometimes over 50 million spores that have the capacity for long-distance dispersal over thousands of kilometers (Kreulen 1972; van Zanten 1978). Producing abundant air-borne diaspores would appear to guarantee a wide distribution of many bryophyte species (Schofield and Crum 1972). In contrast, extreme geographical isolation such as on oceanic islands affects diversification and speciation, even though bryophyte species have the capability for long-distance dispersal (Oguri et al. 2008). Therefore, oceanic islands may provide models for research on patterns and processes of bryophyte evolution and speciation.

The Bonin (Ogasawara) Islands are oceanic islands located in the northwestern Pacific Ocean, approximately
1000 km south of Tokyo, Japan (Asami 1970). These islands were formed during the Paleocene and rose above sea level before the middle Pleistocene (Kaizuka 1977; Imaizumi and Tamura 1984). Approximately 300 indigenous species of vascular plants are known from these islands, and their percentage of endemism is estimated to be as high as 40–43% (Kobayashi 1978; Ono et al. 1986). A total of 155 species of bryophytes (48 genera and 81 species of mosses, 33 genera and 74 species of liverworts and hornworts) are currently known from the Bonin Islands (Inoue and Iwatsuki 1969, 1970, 1984; Inoue 1970a,b; Iwatsuki 1985; Furuki et al. 1991). The percentage of bryophyte endemism is approximately 5%, which is much lower than that of vascular plants.

Among bryophyte taxa growing on the Bonin Islands, members of the genus *Leucobryum* Hampe (Leucobryaceae, Musci) have been taxonomically well studied by Yamaguchi (1993) and Oguri et al. (2008). This genus is one of the most widely distributed moss genera, containing several widespread species. According to van der Wijk et al. (1964), it includes approximately 180 species. Among members of *Leucobryum*, *L. juniperoides* (Br.) Müll.Hal. is widely distributed in Asia, Europe, Macaronesia, and Madagascar, whereas *L. glaucum* (Hedw.) Angr. is widely distributed throughout temperate to cool temperate regions in the Northern Hemisphere (Yamaguchi 1993; Vanderpoorten et al. 2003). In contrast, some endemic species are observed on oceanic islands such as the Hawaiian Islands and the Bonin Islands. *Leucobryum pachyphyllum* Müll.Hal. and *L. semannii* (Mitt.) are endemic to the Hawaii Islands (Bartram 1933; Staples et al. 2004), whereas *L. boninense* (Sull. & Lesq.) (Oguri et al. 2008) is restricted to the Bonin Islands.

*Leucobryum boninense* is characterized by its perichaetium terminal on short lateral branches and papillose proration on the abaxial surface of apical parts of leaves (Fig. 1; Yamaguchi 1993). This species seems to be closely related to *L. scaberulum* Cardot based on morphological characters. In fact, *L. scaberulum* was treated as a synonym of *L. boninense* by Yamaguchi (1993).

Molecular phylogenetic analyses of the genus *Leucobryum* have been performed based on sequence data of internal transcribed spacer (ITS) regions of ribosomal DNA and chloroplast *rbcL* gene. The results showed that the endemic species, *L. boninense*, is closely related to *L. scaberulum*, *L. scabrum* Sande Lac., and *L. javense* (Brid.) Mitt. (Oguri et al. 2003, 2008). All three species are widely distributed; *L. javense* is widely distributed in East and Southeast Asia, and *L. scabrum* and *L. scaberulum* occur in East Asia (Yamaguchi 1993). Nevertheless, two previous molecular phylogenetic studies did not include plant samples from various parts of the distribution areas and were performed based only on ITS and *rbcL* DNA sequence regions. Therefore, detailed phylogenetic relationships among *L. boninense* and its related species remain poorly understood. *matK*, encoding a splicing-associated maturonase in the land plant chloroplast genome, is a very popular region for phylogenetic study and has been extensively applied to reconstruct angiosperm phylogeny (Rev. Müller et al. 2006). However, the utility of *matK* in bryophyte phylogeny is largely unknown. Only one molecular phylogenetic study has been conducted by Long et al. (2000), but it was based on partial *matK* sequence data.

In this study, we collected *L. boninense* samples and those of its related taxa from various parts of their distribution and performed molecular phylogenetic studies to clarify the phylogenetic relationships among *Leucobryum* species and to infer the origin of *L. boninense*, which is restricted to the Bonin Islands. Phylogenetic trees were constructed based on the combined nucleotide sequences of *rbcL*, *rps4*, 5′ *trnK* intron, *matK*, 3′ *trnL* intron, *trnL-F* intergenic spacer, and ITS regions. Moreover, we verified amplification of the *matK* region for six moss species, in addition to the *Leucobryum* species and obtained their sequence data using six primers including four new internal primers designed in this study.

### Materials and methods

#### Plant materials

Fifty samples belonging to 15 species of *Leucobryum* were collected from Asia and the Pacific regions (Table 1). *Leucobryum sanctum* (Brid.) Hampe was used as an outgroup for the phylogenetic analysis, based on a previous molecular phylogenetic study of the entire genus by Oguri et al. (2003). Six additional moss species of different...
| Taxon                  | Voucher specimen | Origin of sample                  | rbcL   | rps4   | trnK intron | trnL-F | ITS            |
|-----------------------|------------------|-----------------------------------|--------|--------|-------------|--------|----------------|
| **Leucobryum aduncum** | 1 HIRO 140862    | Indonesia. Borneo                 | A8124781* | A8740043 | A8742458    | A8742374 | A8125287*     |
|                       | 2 HIRO 140934    | Indonesia. Borneo                 | A8739623 | A8740044 | A8742459    | A8742375 | A8763349      |
|                       | 3 HIRO 138507    | Malaysia. Malay Pen.              | A8739624 | A8740045 | A8742460    | A8742376 | A8763350      |
|                       | 4 HIRO 166266    | Sri Lanka. Nuara                  | A8739625 | A8740046 | A8742461    | A8742377 | A8763351      |
|                       | 5 HIRO 166267    | Sri Lanka. Nuara                  | A8739626 | A8740047 | A8742462    | A8742378 | A8763352      |
|                       | 6 HIRO 166239    | Vanuatu                           | A8739627 | A8740048 | A8742463    | A8742379 | A8763353      |
|                       | 7 HIRO 166241    | U. S. A. Florida                  | A8124784* | A8740049 | A8742464    | A8742380 | A8125288*     |
| **L. albidum**         | 1 MAK B119207    | Japan. Ogasawara Isls. Chichijima Is. | A8739629 | A8740050 | A8742465    | A8742381 | A8763354      |
|                       | 2 MAK B119201    | Japan. Ogasawara Isls. Hahajima Is. | A8739630 | A8740051 | A8742466    | A8742382 | A8763355      |
|                       | 3 MAK B119184    | Japan. Ogasawara Isls. Anijima Is. | A8739631 | A8740052 | A8742467    | A8742383 | A8763356      |
|                       | 4 MAK B119190    | Japan. Ogasawara Isls. Anijima Is. | A8739632 | A8740053 | A8742468    | A8742384 | A8763357      |
|                       | 5 MAK B119192    | Japan. Ogasawara Isls. Anijima Is. | A8739633 | A8740054 | A8742469    | A8742385 | A8763358      |
|                       | 6 HIRO 268806    | Japan. Ogasawara Isls. Kita-ivo Is. | A8739634 | A8740055 | A8742470    | A8742386 | A8763359      |
|                       | 7 HIRO 269656    | Japan. Ogasawara Isls. Kita-ivo Is. | A8739635 | A8740056 | A8742471    | A8742387 | A8763360      |
| **L. bowringi**        | HIRO 139187      | Japan. Yakushima Isl.             | A8124790* | A8740057 | A8742472    | A8742388 | A8125290*     |
|                       | HIRO 203728      | New Zealand                       | A8288196** | A8740058 | A8742473    | A8742389 | A8285170**    |
| **L. candidum** (F. Beauv.) | HIRO 140710    | Indonesia. Borneo                 | A8124792* | A8740059 | A8742474    | A8742390 | A8125291*     |
|                       | 2 HIRO 140820    | Indonesia. Borneo                 | A8739636 | A8740060 | A8742475    | A8742391 | A8763361      |
|                       | 3 MAK B119208    | Philippines                       | A8739637 | A8740061 | A8742476    | A8742392 | A8763362      |
| **L. chlorophyllosum** | HIRO 138407      | Japan. Hokkaido                    | A8124788* | A8740062 | A8742477    | A8742393 | A8125292*     |
| Müll. Hal.            |                 |                                    |         |        |             |        |                |
| **L. glaucum**         | HIRO 120786      | Japan. Amami-oshima Isl.          | A8739638 | A8740063 | A8742507    | A8742394 | A8194567      |
| (Hedw.) Ångstr.        | 2 MAKX119211     | Japan. Amami-oshima Isl.          | A8739639 | A8740064 | A8742479    | A8742395 | A8763363      |
|                       | 3 HIRO 120264    | Taiwan. Pingtung County           | A8124791* | A8740065 | A8742480    | A8742396 | A8125294*     |
|                       | 4 HIRO 138505    | Malaysia. Malay Pen.              | A8739640 | A8740066 | A8742481    | A8742397 | A8763364      |
|                       | 5 HIRO 138508    | Malaysia. Malay Pen.              | A8739641 | A8740067 | A8742482    | A8742398 | A8763365      |
|                       | 6 HIRO 166240    | Thailand. Doi Inthanon            | A8739642 | A8740068 | A8742483    | A8742399 | A8763366      |
|                       | 7 HIRO 166247    | Malaysia. Borneo                  | A8739643 | A8740069 | A8742484    | A8742400 | A8763367      |
|                       | 1 HIRO 139224    | Japan. Yakushima Isl.             | A8124786* | A8740070 | A8742485    | A8742401 | A8125295*     |
| **L. juniperoides**    | HIRO 119467      | Hawaii. Oahu Isl.                 | A8124782* | A8740071 | A8742486    | A8742402 | A8125296*     |
| (Br.) Müll. Hal.       | HIRO 140948      | Indonesia. Borneo                 | A8124787* | A8740072 | A8742487    | A8742403 | A8125297*     |
|                       | 1 HIRO 136706    | Hong Kong. Lantau Isl.            | A8288199** | A8740073 | A8742488    | A8742404 | A8285178**    |
|                       | 2 HIRO 136707    | Hong Kong. New Territories        | A8739644 | A8740074 | A8742489    | A8742405 | A8285179**    |
|                       | 3 MAK B119196    | Hong Kong. New Territories        | A8739645 | A8740075 | A8742490    | A8742406 | A8763368      |
|                       | 4 MAK B119194    | China. Guandong Province          | A8739646 | A8740076 | A8742491    | A8742407 | A8763369      |

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genera were also included in our analyses to conduct polymerase chain reaction (PCR) amplification of trnK intron including matK and to obtain their sequence data: *Tetraphis pellucida* Hedw. (Tetraphidaceae), *Brothera leana* (Sull.) Müll. Hal. (Dicranaceae), *Dicranodontium denudatum* (Brid.) E.G. Britt. ex Williams (Dicranaceae), *Hypnum plumaeforme* Wilson (Hypnaceae), *Isopterygium propaguliferum* Toyama (Hypnaceae), and *Rhytidium rugosum* (Hedw.) Kindlb. (Hylocomiaceae) (Appendix S1). Voucher specimens are deposited at Herbarium of Hiroshima University, Hiroshima, Japan (HIRO) or Makino Herbarium (MAK), Tokyo Metropolitan University, Tokyo, Japan.

### DNA extraction, PCR amplification, and sequencing

Total DNA was extracted either from fresh samples or dried herbarium specimens using the phenol-chloroform method of Tsubota et al. (2002) with some modifications. Six cpDNA regions, *rbcL*, *rps4*, *5′ trnK intron*, matK, *3′ trnK intron*, and *trnL-F* intergenic spacer and one nrDNA region, ITS were amplified by PCR using a thermal cycler (Table 2). Each fragment was amplified with PrimeSTAR Max DNA Polymerase (TaKaRa Bio, Otsu, Shiga, Japan) using 10 μl reactions volumes in a thermal cycle with the following conditions: 98°C for 30 sec followed by 30 cycles of 98°C for 10 sec, 55°C for 5 sec, 72°C for 30 sec and 72°C for 30 sec. After confirming PCR amplification on a 1.0% agarose gel, the amplified products were incubated at 37°C for 30 min and 80°C for 20 min with ExoSAP-IT (usb, Cleveland, OH, USA) to remove any excess primers and nucleotides. Eight primers for *rbcL*, two primers for *rps4*, six primers for *trnK* intron including *matK*, two primers for *trnL-F*, and five primers for ITS were used for the cycle sequencing reactions (Table 2) with an ABI PRISM BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA). The sequencing reaction products were purified, concentrated by ethanol precipitation with sodium acetate and their nucleotide sequences were determined using an automated DNA sequencer (ABI PRISM 3100, Applied Biosystems). The obtained sequences were submitted to the DDBJ database (Table 1 and Appendix S1).

### Phylogenetic analysis

We obtained *rbcL* sequence data of 14 samples belonging to 13 taxa and ITS sequence data of 21 samples belonging

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**Table 1.** Continued.

| Taxon | Voucher specimen | Origin of sample | rbcL | rps4 | trnK intron | trnL-F | ITS |
|-------|------------------|------------------|------|------|-------------|--------|-----|
| L. scabrum Sande Lac. | MAK B119193 Japan. Wakayama-ken | | | | | | AB763371 |
| | 2 HIRO 139186 Japan. Yakushima Isl. | | | | | | AB124793* AB740085 AB742500 AB742416 AB125298* |
| | 3 MAK B119202 Japan. Amami-oshima Isl. | | | | | | AB739654 AB740086 AB742501 AB742417 AB763372 |
| | 4 MAK B119210 Japan. Amami-oshima Isl. | | | | | | AB739655 AB740087 AB742502 AB742418 AB763373 |
| | 5 HIRO 218554 Japan. Okinawa Isl. | | | | | | AB739656 AB740088 AB742503 AB742419 AB763374 |
| | 6 HIRO 120226 Taiwan. Pingtung County | | | | | | AB739657 AB740089 AB742504 AB742420 AB763375 |
| | 7 HIRO 120156 Taiwan. Taichung County | | | | | | AB739649 AB740079 AB742494 AB742410 AB285180** |
| | 8 HIRO 120158 Taiwan. Taichung County | | | | | | AB739650 AB740080 AB742495 AB742411 AB763370 |
| | 9 HIRO 136709 Hong Kong. New Territories | | | | | | AB739658 AB740090 AB742505 AB742421 AB763376 |
| L. seemannii Mitt. | HIRO 119505 Hawaii. Maui Isl. | | | | | | AB739659 AB740091 AB742508 AB742422 AB285183** |
| L. sumatranum (Brid.) Hampe ex M.Reisch. | HIRO 166243 Malaysia. Borneo | | | | | | AB124785* AB740092 AB742506 AB742423 AB125299* |

*Oguri et al. 2003  **Oguri et al. 2008  

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to 14 taxa of the genus *Leucobryum* from the DNA database. The obtained *rbcL*, *rps4*, 5′ *trnK* intron, *matK*, 3′ *trnK* intron, *trnL*-F, and ITS sequences were separately aligned using the program MUSCLE (Edgar 2004).

We performed the Incongruence Length Difference (ILD) test (Farris et al. 1995) implemented in PAUP* version 4.0 beta (Swofford 2002) before phylogenetic reconstruction to confirm topological congruence between each DNA region. One hundred partition homogeneity replicates were implemented in the test using the heuristic search option with 100 random addition sequences. And then, we performed molecular phylogenetic analyses with combined all six chloroplast DNA plus one nuclear DNA sequences. When these analyses were carried out, identical sequences were pruned to include only one representative from each species. Therefore, a total of 35 operational taxonomic units, including outgroup, were used for the following analyses.

Bayesian inference (BI) analysis was performed using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). The best-fitting model for nucleotide substitution was selected for the combined seven regions based on Akaike information criterion (Akaike 1974) implemented in MrModeltest 2.2 (Nylander 2004), and GTR + I + G model was chosen. The analysis was performed for 1,000,000 generations with four chains, with samples taken every 100 generations.

Maximum likelihood (ML) analysis was conducted with PAUP* 4.0b10 using the best-fitting model GTR + I + G chosen by MrModeltest 2.2. A heuristic search algorithm was engaged with 100 random addition replicates and tree-bisection-reconnection (TBR) branch-swapping, and MulTrees on. The ML bootstrap value were computed in PAUP* 4.0b10 by running 1000 replicates with a full heuristic search using 100 random addition sequences, TBR branch-swapping, and MulTrees off (holding one tree at each step).

Maximum parsimony (MP) analysis was performed using PAUP* 4.0b10. A heuristic search algorithm was engaged with 100 random addition replicates and TBR branch-swapping, and MulTrees on. Parsimony bootstrap values were calculated using PAUP* 4.0b10. The bootstrap analysis used 1,000 bootstrap replicates, the heuristic search algorithm, 100 random addition sequences, TBR branch-swapping, and MulTrees off (holding one tree at each step).

### Results

#### Sequence characteristics

Table 3 summarizes the sequence information for all *rbcL*, *rps4*, partial 5′ *trnK* intron, *matK*, partial 3′ *trnK* intron, *trnL*-F, and ITS regions, including the length of each

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Table 2. PCR primers used in this study.

| Analyzed region | Primer name | Sequence | References |
|-----------------|-------------|----------|------------|
| *rbcL*          | atbB175R    | TGT TGA ACT TCA CAA GTA ACA | Manhart 1994 |
|                 | rbcL 256    | GCT ATG ATC TTA CAA GAG CAG | Tsubota et al. 2000 |
|                 | rbcL 549    | TGT CTT GTG GGT GGA C | Tsubota et al. 1999 |
|                 | rbcL 919G   | CAT GGT ATG CAT TTC GTG GTA | Tsubota et al. 2001 |
|                 | rbcL 600R   | GTG AAA TCA AGT CCA CCA CG | Tsubota et al. 1999 |
|                 | rbcL 1098R  | AAC ACC TGG TAA AGA AAC C | Tsubota et al. 1999 |
|                 | rbcL 1346hR | GCA GCT AAT TCA GGA CTA GGA CTC C | Tsubota et al. 1999 |
| *trnK* intron   | *trnK* [tRNA-Lys(UAA)exon1](including *matK*) | CCG ACT AGT GCC GGT TAC GGA CCA ACC | Demesure et al. 1995 |
|                 | *trnK* aF   | ARW TTC ATCCAA ACC ATT GAC AAG G | Designed this study |
|                 | *trnK* 410F | TAT CAA TCT ATT CAT TCT GTA TTT CCT TTT | Designed this study |
|                 | *trnK* 410R | AAA AGG AAA TAC RGA ATG AAT AGA TGG ATA | Designed this study |
|                 | *trnK* aR   | ATT GCA CAC GCC TTT TTC TAT GT | Designed this study |
|                 | *trnK* [tRNA-Lys(UAA)exon2] | CAA CGG TAG AGT CAG CTG TTA | Demesure et al. 1995 |
| *trnL*-F        | c           | CGA AAT CGG TAG ACC GAG AG | Taberlet et al. 1991 |
|                 | f           | ATT TGA ACT GGT GAC ACC AG | Taberlet et al. 1991 |
| *ITS*           | 18S1659B    | CGT CGC TCC TAC CGA TTG | Oguri et al. 2003 |
|                 | 18S1764B    | AGA GGA AGG AGA AGT CGT AAC | Oguri et al. 2003 |
|                 | 5.8S10B     | CTC AGC AAC GGA TAT CTT GG | Oguri et al. 2003 |
|                 | 26S102BR    | CCG GTT CGC TCG CGG | Oguri et al. 2003 |
|                 | 26S166BR    | GAG GAC GCT TCT CGA GAC TAC | Oguri et al. 2003 |

PCR amplification primers are shown in bold.
region, numbers of variable and parsimony-informative sites, number of most parsimonious trees, tree length, consistency index (CI), and retention index (RI).

The ILD test did not detect incongruence between each pair of DNA data sets tested (combined data of the seven regions: \textit{rbcL} + \textit{rps4} + 5’ \textit{trnK} intron + \textit{matK} + 3’ \textit{trnK} intron + \textit{trnL-F} + \textit{ITS}, \( P = 0.01 \); other data not shown). Based on these results, we combined all seven DNA sequences into one large data set, and the obtained phylogenetic results based on the combined data are shown (Table 3). The total aligned length for the combined sequences was 5,240 characters and 531 (10.9%) characters were parsimony informative. Parsimony analysis of all seven DNA sequences into one large data set, and the obtained phylogenetic results based on the combined data are shown

|                | \textit{rbcL} | \textit{rps4} | 5’ \textit{trnK} intron | \textit{matK} | 3’ \textit{trnK} intron | \textit{trnK} intron | \textit{trnL-F} |
|----------------|--------------|--------------|-------------------------|--------------|-------------------------|---------------------|----------------|
| Aligned length (bp) | 1428         | 471          | 322                     | 1524         | 136                     | 1982                | 439 |
| bp included in analyses | 1428         | 467          | 316                     | 1521         | 132                     | 1969                | 429 |
| Variable characters | 52 (3.6%)    | 24 (5.1%)    | 33 (10.4%)              | 129 (8.5%)   | 15 (11.4%)              | 177 (9.0%)          | 37 (8.6%) |
| Parsimony-informative chars. | 34 (2.4%)    | 15 (3.2%)    | 21 (6.6%)               | 87 (5.7%)    | 7 (5.3%)                | 115 (5.8%)          | 26 (6.1%) |
| Number of trees (MP) | 1            | 4            | 1                       | 960          | 1                       | 318                 | 37  |
| Tree length | 69           | 28           | 38                      | 164          | 17                      | 221                 | 45  |
| CI            | 0.783        | 0.857        | 0.947                   | 0.787        | 1.00                    | 0.824               | 0.844 |
| RI            | 0.885        | 0.913        | 0.971                   | 0.904        | 1.00                    | 0.916               | 0.936 |

Table 3. Phylogenetic features of obtained nucleotide sequences of cpDNA and nrDNA in this study.

|                | \textit{rbcL} | \textit{rps4} | 5’ \textit{trnK} intron | \textit{matK} | 3’ \textit{trnK} intron | \textit{trnK} intron | \textit{trnL-F} |
|----------------|--------------|--------------|-------------------------|--------------|-------------------------|---------------------|----------------|
| Aligned length (bp) | 920          |              |                         |              |                         |                     | 5240 |
| bp included in analyses | 589          |              |                         |              |                         |                     | 4882 |
| Variable characters | 241 (40.9%)  |              |                         |              |                         |                     | 730 (15.0%) |
| Parsimony-informative chars. | 151 (25.6%)  |              |                         |              |                         |                     | 531 (10.9%) |
| Number of trees (MP) | 1            |              |                         |              |                         |                     | 2   |
| Tree length | 392           |              |                         |              |                         |                     | 1120 |
| CI            | 0.778        |              |                         |              |                         |                     | 0.768 |
| RI            | 0.894        |              |                         |              |                         |                     | 0.862 |

Cl = Consistency index; RI = Retention index.

The total aligned length for the combined sequences was 5,240 characters and 531 (10.9%) characters were parsimony informative. Parsimony analysis of all seven data regions resulted in two MP trees (Tree length = 1120, CI = 0.768, RI = 0.862).

We also tested the utility of \textit{matK} for resolving phylogenetic relationships among \textit{Leucobryum} species. The total aligned length for \textit{trnK} intron including \textit{matK} was 1,969 characters and 115 (5.8%) characters were parsimony informative. A percentage of parsimony-informative characters of \textit{trnK} intron sequence data was significantly higher than that of other chloroplast sequence data (\textit{rbcL}: 34 characters, 2.4%; \textit{rps4}: 15 characters, 3.2%), except for \textit{trnL-F} sequence data (26 characters, 6.1%).

We sequenced the chloroplast \textit{trnK} intron including \textit{matK} from six additional moss species of other genera including \textit{Tetraphis pellucida} (Tetraphidaceae), \textit{Brothera leana} (Dicranaceae), \textit{Dicranodontium denudatum} (Dicranaceae), \textit{Hypnum plumaeforme} (Hypnaceae), \textit{Isopterygium propaguliferum} (Hypnaceae), and \textit{Rhytidium rugosum} (Hylocomiaceae) (Appendix S1). The region was not amplified for the Hepaticae and Anthocerotae plant materials when we used PCR primers for exon 1 and exon 2 of the \textit{trnK} intron (Demesure et al. 1995; see also Table 2).

Phylogenetic analyses

Figure 2 shows a majority rule consensus tree generated by BI analysis. The major five clades recognized in the analyses are indicated with Roman numerals (I–V). These clades were supported by high statistical values. Clade I contained \textit{L. bowringii} Mitt. and \textit{L. sumatranum} (Brid.) Hampe ex M.Fleisch., and clade II contained \textit{L. albidum} (P.Beauv.) Lindb., \textit{L. glaucum}, and \textit{L. juniperoideum}. Clades I and II were supported by high statistical support (Bayesian posterior probabilities/ML bootstrap/MP bootstrap = 1.00/100/100). Clade III contained only one species, \textit{L. chlorophyllosum} M€ull.Hal., from the Philippines and Indonesia. Clade IV contained \textit{L. candidum} (Brid. ex Beauv.) and \textit{L. aduncum} Dozy & Molk.. All three species contained in Clades III and IV are distributed in southeastern Asia and the south Pacific region. Clade V contained six species: \textit{L. boninense} restricted to the Bonin Islands, \textit{L. javense},
**Figure 2.** Molecular phylogenetic tree of *Leucobryum* species inferred from combined sequence data from seven regions including *rbcL*, *rps4*, the 5′ *trnK* intron, *matK*, the 3′ *trnK* intron, *trnL-F*, and ITS. Bayesian posterior probabilities (BI), maximum likelihood bootstrap probabilities (ML), and maximum parsimony bootstrap probabilities (MP) are shown on each branch as (BI/ML/MP). Support values <50% are shown as hyphens (-). Scale bar indicates a branch length corresponding to 0.1 substitutions per site.

*L. scabrum*, *L. scaberulum*, and *L. pachyphyllum* from Oahu Island, and *L. seemannii* from Maui Island.

*Leucobryum boninense* from various islands in the Bonin Islands made a clade with strong statistical support (Bayesian posterior probabilities/ML bootstrap/MP bootstrap = 1.00/99/99), and was closely related to *L. scabrum* from Japan, Taiwan, and Hong Kong, and *L. javense* from Japan. Among the *L. boninense* samples, those from the Ogasawara Islands (Chichijima Island, Hahajima Island, and Anijima Island) and Kita-iwo Island showed a 1-bp difference in the *rbcL*, 1-bp deletion in the 5′ *trnK* intron, and 10-bp deletion in the ITS. The sequences of the *rps4*,
Three species, _L. scabrum_, _L. scaberulum_, and _L. javense_ showed similar sequences to that of _L. boninense_. Our phylogenetic results showed that the plant samples of _L. scabrum_ and _L. scaberulum_ were monophyletic, in contrast to those of _L. javense_ which were polyphyletic. _Leucobryum scaberulum_ contained two different groups: the Ryukyus group consisting of plant materials from the Ryukyus and Taiwan and the China group consisting of those from Hong Kong and Guangdong. _Leucobryum javense_ was divided into four clades, samples #1 and 2 from Japan were closely related to _L. boninense_ and _L. scabrum_, sample #4 from Malaysia was sister to the Hawaiian endemic species, _L. pachyphyllum_ and _L. seemannii_, samples #3, 5, and 6 were sister to _L. scaberulum_, and sample #7 from Malaysia formed an independent clade.

**Discussion**

**Origin of Leucobryum boninense, endemic to the Bonin Islands, Japan**

In this study, the endemic species _L. boninense_ formed a robust clade with five related species including _L. scabrum_, _L. javense_, _L. scaberulum_, _L. pachyphyllum_, and _L. seemannii_, as suggested by Oguri et al. (2003, 2008) (Fig. 2; clade V), and was closely related to _L. scabrum_ from Japan, Taiwan, and Hong Kong and _L. javense_ from Japan. No differences in the _rps4_ sequences were observed between the _L. boninense_ samples and those of _L. scabrum_, in contrast, only 1-bp difference was observed in the _rps4_ sequences between the _L. boninense_ samples and those of _L. javense_ from Japan. In the _rbcL_ sequences, 1-bp difference was observed between the _L. boninense_ samples from the Ogasawara Islands (Chichijima Island, Hahajima Island, and Anijima Island) and those of _L. boninense_ from Kita-iwo Island, as well as between those of _L. boninense_ from the Ogasawara Islands and those of _L. scabrum_. _Leucobryum boninense_ samples from the Ogasawara Islands and _L. javense_ from Japan had the same _rbcL_ sequences. In morphological characters, Yamaguchi (1993) mentioned that _L. boninense_ is morphologically similar in the absence of a central strand in the stem and perichaetial terminal on short lateral branches to _L. scabrum_ and _L. javense_. However, this species is clearly distinguishable from _L. scabrum_ based on leaves being papillose-prorate on the abaxial surface, and is also clearly distinguishable from _L. javense_ based on its small plant size (Yamaguchi 1993). Therefore, this molecular phylogenetic result suggests that _L. boninense_, which is restricted to the Bonin Islands, originated from Japan, Taiwan, or Hong Kong. The bryophyte flora of the Bonin Islands is generally regarded as similar to that of East and Southeast Asia (Iwatsuki 1985). However, this is still the first demonstration that molecular phylogenetic data directly support an East Asian origin of a moss species endemic to the Bonin Islands.

**Origin of the Hawaiian endemic species of Leucobryum**

In the case of Hawaiian mosses, their geographical origins remain unclear, although it is known that that Hawaiian moss flora, especially of cosmopolitan taxa, shows almost no connection with those of the American continents (Bartram 1933). _Leucobryum pachyphyllum_ and _L. seemannii_ are endemic to the Hawaiian Islands, and the two species are morphologically characterized by medium-sized plants and abaxially rough leaves (Bartram 1933; Staples et al. 2004). Our phylogenetic tree showed that the two species formed a monophyletic group, and were closely related to _L. javense_ from Malaysia (Fig. 2). _Leucobryum albidum_, which is restricted in North America, formed a clade with _L. glaucum_ from Japan and _L. juniperoides_ from Japan, and is genetically distinct from the Hawaiian _Leucobryum_ (Fig. 2; clade II). This species is clearly distinguished from the Hawaiian endemic species by smooth abaxial leaf surface and terminal perichaetia on stems (Bartram 1933). Molecular phylogenetic results suggested that the two Hawaiian endemic species may be originated from a southeastern Asian, not from the America.

**Utility of the chloroplast matK gene for resolving phylogenetic relationships among Leucobryum species**

Bryophyte phylogeny and biogeography have been studied using nucleotide sequence information of nuclear and plastid DNAs such as those of nuclear ITS regions, chloroplast _rbcL_, _rps4_, _trnG_ and _trnL-F_, for resolving origin and species delimitation (e.g. Huttunen et al. 2008; Oguri et al. 2008; Shaw et al. 2008; Preußing et al. 2010; Villarreal et al. 2010). However, phylogenetic analyses using chloroplast _matK_ have not been well performed yet in bryophytes, although this gene is a powerful source for angiosperm phylogenetic analyses (Rev. Müller et al. 2006). A molecular phylogenetic study of Asterella (Aytioniaceae, Marchantiopsida), inferred from partial _matK_ sequences (aligned length = 759 bp) by Long et al. (2000), is the only study to date. Their phylogenetic analysis strongly supported monophyly of Aytioniaceae; therefore, they concluded that the _matK_ region is a useful source of phylogenetic signals in Asterella and related marchantioid liverworts. In the present study, we compared useful sequence information among each sequence data for 50 samples containing 15 species of _Leucobryum_ (Table 3). A percentage of parsimony-infor-
mative characters in the trnK intron (5.8%) was significantly higher than other chloroplast DNA regions, rbcL and rps4, although its percentage in the ITS (25.6%) was the highest among the seven regions. Maximum parsimony trees based on the trnK intron sequence data (CI = 0.824, RI = 0.916) were relatively robust than those based on the rbcL (CI = 0.783, RI = 0.885), ITS (CI = 0.778, RI = 0.894), and the combined seven sequence data (CI = 0.768, RI = 0.862). Therefore, the sequence data of trnK intron region including matK provided more informative signals for phylogenetic reconstruction among Leucobryum species.

In the present study, we also sequenced the chloroplast trnK intron region including matK of six moss species from various taxonomic groups (Appendix S1). Among these six moss species, Brothera leana and Dicranodontium denudatum were mostly closely related to Leucobryum species, whereas the remaining four species had largely different rbcL sequences from Leucobryum species, according to the results of a previous molecular phylogenetic study by Tsubota et al. (2004). Therefore, six primers (four of the six were newly designed in the present study, Table 2) for the trnK intron and matK are expected to be useful for molecular phylogenetic analyses in various moss taxa.

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Author contributions

E.O., T.Y., H.T., H.D., and N.M. designed the study; E.O. and T.Y. performed the sampling; E.O. analyzed the data; E.O. and N.M. wrote the manuscript.

Biosketches

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Conflict of Interest

None declared.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Six moss species analyzed in this study, their voucher information, and GenBank accession numbers of the DNA sequences.