Use of Length Heterogeneity Polymerase Chain Reaction (LH-PCR) as Non-Invasive Approach for Dietary Analysis of Svalbard Reindeer, *Rangifer tarandus platyrhynchus*

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Abstract

To efficiently investigate the forage preference of Svalbard reindeer (*Rangifer tarandus platyrhynchus*), we applied length-heterogeneity polymerase chain reaction (LH-PCR) based on length differences of internal transcribed spacer (ITS) regions of ribosomal RNA (rRNA) to fecal samples from *R. tarandus platyrhynchus*. A length-heterogeneity (LH) database was constructed using both collected potential food sources of Svalbard reindeer and fecal samples, followed by PCR, cloning and sequencing. In total, eighteen fecal samples were collected between 2011 and 2012 from 2 geographic regions and 15 samples were successfully amplified by PCR. The LH-PCR analysis detected abundant peaks, 18.6 peaks on an average per sample, ranging from 100 to 500 bp in size and showing distinct patterns associated with both regions and years of sample collection. Principal component analysis (PCA) resulted in clustering of 15 fecal samples into 3 groups by the year of collection and region with a statistically significant difference at 99.9% level. The first 2 principal components (PCs) explained 71.1% of the total variation among the samples. Through comparison with LH database and identification by cloning and sequencing, lichens (*Stereocaulon* sp. and *Ochrolechia* sp.) and plant species (*Salix polaris* and *Saxifraga oppositifolia*) were detected as the food sources that contributed most to the Svalbard reindeer diet. Our results suggest that the use of LH-PCR analysis would be a non-invasive and efficient monitoring tool for characterizing the foraging strategy of Svalbard reindeer. Additionally, combining sequence information would increase its resolving power in identification of foraged diet components.

Introduction

Svalbard reindeer (*Rangifer tarandus platyrhynchus*) lives on the high-arctic archipelago of Svalbard (74°–80° N lat.) where snow and ice cover most of the local vegetation for 8 months of the year [1–3]. Because of the long winter period resulting in relatively lower forage availability and poor food quality, the reindeer have to replenish fat reserves for winter hibernation and development during the summer period [4–7]. In addition, extreme seasonal variations in the high-arctic region impose strong pressures on arctic herbivores to feed on vegetation in a highly efficient manner to satisfy their energy requirements [7]. Previous studies have reported that reindeer are highly selective feeders and prefer lichens, mosses, graminoids, and various other plant species as food sources in the summer period [8,9]. These preferences might be associated with their special nutritional needs (food quality) or plant biomass represented as food quantity [7,10].

To investigate forage preference of Svalbard reindeer, previous studies have used either directly observed feeding behaviors by tracking the reindeer or analyzed diet composition from undigested contents in feces or rumen sampled in killed reindeer [4,7,10]. In general, these approaches have the advantage of providing direct information about diet and differentiating forage preferences by age and sex. In addition, directly assessing food consumption irrespective of food digestibility could be an obvious advantage of this approach. However, the direct-observation approach is very laborious because it allows monitoring of only a limited number of individuals at one time. Likewise, analysis of rumen contents requires direct handling of reindeers after killing and identification of undigested remains, which are difficult, laborious, and time consuming to perform.

Recently, molecular approaches have been widely used as non-invasive methods to study animal diet from feces or food remnants [11–15]. However, most of these approaches required laborious and time-consuming conventional sequencing methods, including cloning of PCR products and individual sequencing of clones [16]. The recent development of next-generation sequencing (NGS) methods has enabled the increased use of NGS-based methods on fecal samples to analyze diets [16–20]. The use of NGS technology for dietary analysis can provide an unprecedented amount of sequence data at lower costs than conventional molecular methods [21]. However, in spite of the advantages, application of NGS technology for reindeer diets may be pricy because available and preferred food sources for reindeer are limited at a given time and...
each fecal sample would contain only several short-term dietary compositions [8-9].

Applications of length-heterogeneity analysis by PCR (LH-PCR) to study forage preference on the basis of fecal samples can solve various difficulties typically encountered in conventional sampling methods. LH-PCR is widely applied, for example, to study the microbial diversity in natural ecosystems, and LH-PCR has been demonstrated to be an easy, fast, reliable, and highly reproducible method [22–26]. LH-PCR is capable of discriminating amplicons originating from different organisms on the basis of natural variation in the lengths of its DNA target regions [26]. Each peak in LH profiles represents distinct genotypes contributing to diet composition; peak numbers correspond to minimum richness of diet genotypes; and peak heights indicate abundance of each genotype [27].

Our study aimed to evaluate the potential applicability of an LH-PCR approach for dietary analysis of R. tarandus platyrhynchos. First, we constructed an LH-length database of potential food sources, including various vascular plants, mosses, lichens, and mushrooms representing the local flora in Svalbard. Second, we conducted LH-PCR of reindeer fecal samples collected from different sites in 2011 and 2012. Third, we determined the forage preference of Svalbard reindeer by comparing the LH-PCR profiles of collected fecal samples with the profiles in the newly constructed LH-length database.

Materials and Methods

A. Study sites and sample collection

Our study was performed in Ny-Alesund (78°53′–78°55′N, 11°46′–12°11′E), located at the northwest coast of Spitsbergen Island, Svalbard in Norway (Fig. 1), with a permission from the Governor of Svalbard and registered in Svalbard Science Forum (www.rcn.no/ssf; RIS ID: 4985). There are small research stations Island, Svalbard in Norway (Fig. 1), with a permission from the Governor of Svalbard and registered in Svalbard Science Forum (www.rcn.no/ssf; RIS ID: 4985). There are small research stations restricted (Fig. 1). All fecal samples were collected only on the approved area near the Dasan Station. In this region, reindeer (R. tarandus platyrhynchos) are protected from hunting or any other human development such as tourism [1,3]. The local vegetation includes short-growing plants, such as mosses, lichens (Cetraria delisei), the polar willow (Salix polaris), the purple saxifrage (Saxifraga oppositifolia), grasses and sedges, all of which contribute to the diet of Svalbard reindeer [2].

Eighteen fecal samples of R. tarandus platyrhynchos were collected during August in 2011 and 2012 from two different glacier areas: Broggerbreen and Lovénbreen in Ny-Alesund, where tourist visits are restricted (Fig. 1). All fecal samples were collected only on the ground from the approved area near the Dasan Station. In addition, various food sources such as common vascular plants and graminoids, mosses, and lichens were collected to construct an LH-length database and to determine forage preferences from reindeer feces. The fecal samples collected were transferred individually into polyethylene bags and kept at 4°C on ice during delivery to the laboratory. All samples were stored at -20°C until DNA extraction.

B. Sample preparation and DNA extraction

DNA was extracted from feces using the QiAamp DNA Stool Mini Kit (Qiagen) following manufacturer’s protocols except for the lysis step. For sufficient homogenization, we added one or two 5-mm stainless steel beads (Qiagen) in the lysis step and mixed them by shaking on a Mixer Mill (Retsch, Germany) at 20 Hz for 1 min. Extracted DNA was eluted in 200 μl of AE buffer, and dilutions of 1:10 were made in HPLC-grade H2O for use in subsequent PCRs. DNA extracts were stored at -20°C until further analyses.

C. PCR amplification, cloning, and sequencing for construction of LH database

The universal ITS primer set comprising ITS3, 5′-GCATC-GATGAAAGACGGACG-3′ and ITS4, 5′-TCCTCCGCTTATTGATATGC-3′ was used for amplifying the ITS2 region of ribosomal RNA (rRNA) genes [28]. In each PCR amplification, 1 μl of extracted DNA was added to 24 μl of the amplification mixture, resulting in final concentrations of 1× Ex Tag Buffer, 1.5 mM of MgCl2, 0.2 mM of dNTPs, 0.2 μM of each primer, and 1 U of Ex Tag DNA polymerase (Takara, Japan), in a final reaction volume of 25 μl. PCR conditions were as follows: an initial denaturation at 95°C for 5 min, 45 cycles of denaturation at 95°C for 30 s; annealing at 50°C for 30 s; elongation at 72°C for 1 min 30s, and a final extension step at 72°C for 7 min. PCR products amplified in the reaction were purified using Expir PCR SV Kit (GeneAll, Korea). Purified PCR products were ligated into the pGEM-T Easy Vector according to the manufacturer’s protocols (Promega, USA) and transformed into DH5α chemically competent cells. Cells were plated in Luria–Bertani agar + ampicillin medium with 40 μl of X-gal solution (2% w/v) for antibiotic selection and blue-white screening. After the cloning step, 3 to 5 white colonies were selected and used in colony PCR for amplification with M13F and M13R primers. An initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 94°C for 30 s; annealing at 55°C for 30 s; elongation at 72°C for 1 min and a final extension step at 72°C for 7 min. PCR products amplified in the reaction were purified using the Expir PCR SV Kit (GeneAll, Korea). Sequencing was conducted by a commercial sequencing service company (Macrogen, Korea). Each obtained DNA sequence was identified by BLASTN searches of the GenBank database. Sequence alignments and length calculations were conducted using the MEGA 5 program [29].

D. LH analysis

For LH analysis, FAM-ITS3 and ITS4 were used in PCR amplifications. FAM-ITS3 was the ITS3 primer labeled on its 5′ end with the phosphoramidite fluorochrome 5-carboxyfluorescein (FAM). The buffer and reagent composition of each PCR reaction was the same as described above. For PCR cycling, conditions were modified to minimize amplification bias as follows: an initial denaturation at 95°C for 7 min, 30 cycles of denaturation at 95°C for 30 s; annealing at 50°C for 30 s; elongation at 72°C for 1 min 30s, and a final extension step at 72°C for 7 min. Three PCR products amplified under identical conditions were combined and purified as described above. The LH analyses were conducted by a commercial company (Solgent, Korea) with an internal size standard (Genescan 500 ROX, Applied Biosystems) ranging from 55 to 78, which covered most of the major DNA peaks. LH profiles were analyzed using the DAx software (Van Mierlo Software Consultancy, Netherlands).

E. Statistical analysis

LH profiles of collected fecal samples were compiled and aligned to produce a large data matrix (15 observations × 221 peak variables). LH profile data were centered and standardized to relative abundance before conducting principal component analysis (PCA). We assigned “0” when a matching peak was absent. PCA was applied to the weighted covariance data matrix to reduce its dimensionality. Multivariate analysis of variance (MANOVA) was conducted on three groups (Lovénbreen,
Ny-Ålesund in 2011 vs. Brøggerbreen, Ny-Ålesund in 2012 vs.
Lovebreen, Ny-Ålesund in 2012) according to between sampling
years and sites using un-rotated PC scores. Analysis of variance
(ANOVA) was used to test for statistically significant differences of
major foraged components represented as relative peak area
(expressed as %) on first 2 PC-axis, and post hoc tests were
performed by using Tukey’s method (P<0.05). All statistical
analyses were performed with S-Plus 8 for Windows (Insightful
Corp., USA).

Results

A. Construction of LH database from reference samples
and fecal samples

LH lengths were measured from 64 reference samples
containing lichens, mushrooms, and plants and combined with
BLASTN results (Table 1 and 2). LH lengths ranged from 333.4
bp for a lichen species (Ochrolechia sp.) to 494.1 bp for moss
(Polytrichum sp.) (Table 1 and 2). Most of the samples identified as
lichen had LH lengths of <350 bp. Mushrooms had LH lengths
ranging from 369.3 bp for Cortinarius saturninus to 450.3 bp for
Russula silicola, and were divided into three groups by size
difference. Plants and mosses had longer LH lengths, ranging from
364.7 to 494.1 bp, than the other predicted food species. In
addition, 22 unique sequences were detected from collected fecal
samples by selection after cloning (Table 1). Additionally, we
conducted cloning with Sanger sequencing from collected 15 feces
to identify foraged food sources and included in LH database
(Table 2). Twenty-seven different sequences were detected
including lichens (C. delisei and Stereocaulon sp.), mushrooms
(Hebeloma sp. and Cortinarius favrei), and common vascular plants
(Salix polaris and Bistorta vivipara). Several fungi (Cladosporium sp.,
Friedmanniomyces endolithicus, and Thelebolus microsporus) that do not
form mushrooms or lichens were also detected from feces and they
displayed LH lengths ranging from 329.8 to 406.3 bp.

B. LH analysis
Among the 18 fecal samples collected, 15 resulted in successful
PCR amplification, and they were used for LH analysis. Most LH
profiles consisted of an abundant number of peaks, with an
average profile having 18.6 peaks ranging in size from 100 to 500
bp (Fig. 2, Table 3). Most of the informative peaks were detected
in the range of 300 to 500 bp in all LH profiles. The LH profiles
from fecal samples collected in 2011 had higher number of peaks,
39 on an average, than the LH profiles from fecal samples
collected in 2012. However, most peaks from the 2011 samples
showed lower fluorescence intensity than the peaks in the 2012
samples. LH peaks of more than 400 bp in length were detected in
all LH profiles from the 2011 samples. Fecal samples collected in
2012 showed LH profile patterns that were different from the LH
profile patterns of the samples collected in 2011 (Fig. 2). Most of
the LH profiles had the largest peaks at approximately 355 bp, and
they were composed of two major peaks that were shorter than 350 bp.

C. Principal component analysis of LH profiles

PCA of LH profiles was performed to compare dietary components and resulted in the first two principal components (PCs) explaining 71.1% of the total variation among the profiles (PC1 for 53.4% and PC2 for 17.7%; Fig. 3). MANOVA on the PCA scores from the LH-profile comparisons showed that the PCA scores of 3 groups (Love´nbreen, Ny-A˚lesund in 2011 vs. Brøggerbreen, Ny-A˚lesund in 2012 vs. Love´nbreen, Ny-A˚lesund in 2012) were statistically significantly different from each other (P < 0.001). In particular, the PCA score plots showed that fecal samples obtained from different years were separated mainly by the first PC axis. Five of the six peaks that, according to their loading values, represented variables that contribute most to the first principal component, were assigned as lichens (Stereocaulon sp. and Ochrolechia sp.) and vascular plant species (Salix polaris and Saxifraga oppositifolia) on the basis of comparisons with the constructed LH database (Fig. 2 and 3, Table 1 and 2). Plant species were detected only in samples collected in 2011 (59.8% of total relative peak area, Table 3). One-way ANOVA results indicated that the percentage of relative areas for peaks corresponding to lichen species was statistically significantly different among 3 different groups separated on the first PC axis, according to the sampling year and region (P = 0.008).

Table 1. Constructed LH database for 32 vascular plants and 2 bryophyte species living in Ny-Ålesund (78°53′-78°55′N, 11°46′-12°11′E), Spitsbergen Island, Svalbard in Norway.

| Sample ID | Species                        | Amplification length (bp) | Accession | Remarks            |
|-----------|--------------------------------|---------------------------|-----------|--------------------|
| Sval_R1   | Koenigia islandica             | 364.7                     | KC691698  |                    |
| Sval_R2   | Broya glabella sp. purpurascens| 367.5                     | KC691699  |                    |
| Sval_R3   | Cochlearia groenlandica        | 369.9                     | KC691700  |                    |
| Sval_R4   | Draba alpina                   | 370.3                     | KC691701  |                    |
| Sval_R5   | Carex nardina ssp. hepburnii   | 370.6                     | KC691702  |                    |
| Sval_R6   | Oxystria digyna                | 371.8                     | KC691703  |                    |
| Sval_R7   | Cardamine pratensis ssp. angustifolia| 372.7            | KC691704  |                    |
| Sval_R8   | Minuartia biflora              | 384.5                     | KC691705  |                    |
| Sval_R9   | Ranunculus pygmaeus            | 387.2                     | KC691706  |                    |
| Sval_R10  | Ranunculus hyperboreus ssp. arnelli| 388.5                  | KC691707  |                    |
| Sval_R11  | Poa alpina var. vivipara      | 390.9                     | KC691708  |                    |
| Sval_R12  | Festuca sp.                    | 391                       | KC691709  |                    |
| Sval_R13  | Deschampsia alpina             | 391.1                     | KC691710  |                    |
| Sval_R14  | Salix polaris                  | 391.2                     | KC691711  |                    |
| Sval_R15  | Dryas octopetala               | 394.3                     | KC691712  |                    |
| Sval_R16  | Puccinellia vahliana           | 395.2                     | KC691713  |                    |
| Sval_R17  | Cerastium arcticum             | 395.7                     | KC691714  |                    |
| Sval_R18  | Trisetum spicatum ssp. spicatum| 396                       | KC691715  |                    |
| Sval_R19  | Stellaris sp.                  | 396.4                     | KC691716  |                    |
| Sval_R20  | Sagina rivularis               | 397.6                     | KC691717  |                    |
| Sval_R21  | Pedicularis hisruta            | 404                       | KC691718  |                    |
| Sval_R22  | Bistorta vivipara              | 407.9                     | KC691719  |                    |
| Sval_R23  | Cassiope tetragona ssp. tetragona| 408.4               | KC691720  |                    |
| Sval_R24  | Luzula confusa                 | 409.4                     | KC691721  |                    |
| Sval_R25  | Micranthes hieracifolia        | 412.7                     | KC691722  |                    |
| Sval_R26  | Micranthes foliolosa           | 413.9                     | KC691723  |                    |
| Sval_R27  | Saxifraga rivularis ssp. rivularis| 415.3            | KC691724  |                    |
| Sval_R28  | Saxifraga cespitosa            | 417.4                     | KC691725  |                    |
| Sval_R29  | Saxifraga oppositifolia ssp. oppositifolia| 418.5         | KC691726  |                    |
| Sval_R30  | Saxifraga aizoides             | 419.1                     | KC691727  |                    |
| Sval_R31  | Huperzia arctica               | 424                       | KC691728  |                    |
| Sval_R32  | Sanionia uncinata              | 430.5                     | KC691729  | Bryophyte          |
| Sval_R33  | Papaver dahlianum              | 433.7                     | KC691730  |                    |
| Sval_R34  | Polytrichum sp.                | 494.1                     | KC691731  | Bryophyte          |

All of sequences on ITS2 region of rRNA gene were registered in Genbank (Accession number: KC691698-KC691731). They were arranged by the LH length. doi:10.1371/journal.pone.0091552.t001
Table 2. Constructed LH database between 30 different sequences containing collected potential food sources of Svalbard reindeer (*R. tarandus platyrhynchus*) and 27 sequences detected from fecal samples by cloning.

| Sample ID | BLAST results | Amplification length (bp) | Occurrence of identified taxon on Svalbard | Origin of sequence |
|-----------|---------------|---------------------------|-------------------------------------------|------------------|
| **Food sources** | **Description** | **Identities** | **Gaps** | **Occurrence of identified taxon on Svalbard** | **Origin of sequence** |
| Sval_1 | Ochrolechia tartarea (Li) | 286/298(96%) | 1/298(0%) | 333.4 | Ochrolechia sp. |
| Sval_2 | Biatora carneolbida (Li) | 296/297(99%) | 1/297(0%) | 333.7 | Yes |
| Sval_3 | Cetrariella delisei (Li) | 296/297(99%) | 1/297(0%) | 333.8 | Yes |
| Sval_4 | Cetrariella fastigiata (Li) | 292/298(98%) | 2/298(0%) | 333.9 | Cetrariella delisei |
| Sval_5 | Cetrariella fastigiata (Li) | 293/297(99%) | 1/297(0%) | 334 | Cetrariella delisei |
| Sval_6 | Ochrolechia tartarea (Li) | 297/298(99%) | 0/298(0%) | 334.5 | Ochrolechia sp. |
| Sval_7 | Stereocaulon tomentosum (Li) | 293/299(98%) | 0/299(0%) | 335.5 | Stereocaulon sp. |
| Sval_8 | Umbilicaria decussata (Li) | 298/303(98%) | 1/303(0%) | 340.1 | Yes |
| Sval_9 | Umbilicaria umbilicariae (Li) | 296/303(98%) | 0/303(0%) | 340.9 | Umbilicaria sp. |
| Sval_10 | Cladonia arbuscula ssp. beringiana (Li) | 194/194(100%) | 0/194(0%) | 345.4 | Cladonia arbuscula |
| Sval_11 | Cladonia borealis (Li) | 258/258(100%) | 0/258(0%) | 346.9 | Yes |
| Sval_12 | Cladonia grayi (Li) | 311/313(99%) | 0/313(0%) | 349.9 | Cladonia sp. |
| Sval_13 | Cortinarius saturninus (Mu) | 329/329(100%) | 0/329(0%) | 369.3 | Yes |
| Sval_14 | Cortinarius sp. (Mu) | 329/332(99%) | 1/332(0%) | 371.5 | Potentially yes |
| Sval_15 | Hebeloma testaceum (Mu) | 362/363(99%) | 0/363(0%) | 371.8 | Hebeloma sp. |
| Sval_16 | Cladonia arbuscula ssp. beringiana (Mu) | 285/351(99%) | 0/351(0%) | 371.9 | Cladonia arbuscula |
| Sval_17 | Inocybe terrigena (Mu) | 305/357(85%) | 17/357(4%) | 385.4 | Yes |
| Sval_18 | Cortinarius favrei (Mu) | 349/351(99%) | 0/351(0%) | 390.5 | Potentially yes |
| Sval_19 | Cortinarius trivialis (Mu) | 350/351(99%) | 0/351(0%) | 391 | Cortinarius sp. |
| Sval_20 | Salix bebbiana (An) | 359/360(99%) | 0/360(0%) | 391.1 | S. polaris |
| Sval_21 | Cortinarius favrei (Mu) | 350/351(99%) | 0/351(0%) | 391.4 | Yes |
| Sval_22 | Cortinarius favrei (Mu) | 350/351(99%) | 0/351(0%) | 391.6 | Yes |
| Sval_23 | Cortinarius favrei (Mu) | 350/351(99%) | 0/351(0%) | 391.6 | Yes |
| Sval_24 | Entoloma aff. sinuatum (Mu) | 581/583(99%) | 1/583(0%) | 391.7 | Yes |
| Sval_25 | Cortinarius favrei (Mu) | 350/351(99%) | 0/351(0%) | 391.9 | Yes |
| Sval_26 | Salix hetsacea (An) | 358/360(99%) | 0/360(0%) | 392.3 | Yes |
| Sval_27 | Salix bebbiana (An) | 360/360(100%) | 0/360(0%) | 392.6 | S. polaris |
| Sval_28 | Salix bebbiana (An) | 359/359(100%) | 0/359(0%) | 392.6 | S. polaris |
| Sval_29 | Silene paradoxa (An) | 365/367(99%) | 1/367(0%) | 400.5 | Silene sp. |
| Sval_30 | Inocybe leucoloma (Mu) | 360/361(99%) | 0/361(0%) | 401 | Yes |
| Sval_31 | Omphalina chionophila (Mu) | 362/363(99%) | 0/363(0%) | 403 | Yes |
| Sval_32 | Sebacinales (Mu) | 323/366(88%) | 18/366(4%) | 403 | Potentially yes |
| Sval_33 | Omphalina chionophila (Mu) | 362/363(99%) | 0/363(0%) | 403.2 | Yes |
| Sval_34 | Bistorta subscaposa (An) | 374/380(98%) | 0/380(0%) | 409.4 | Bistorta vivipara |
| Sval_35 | Bistorta subscaposa (An) | 372/380(98%) | 0/380(0%) | 410.2 | Bistorta vivipara |
| Sval_36 | Saxifraga fortunei var. alpina (An) | 309/325(95%) | 6/325(1%) | 414.3 | Micranthes hieracifolia |
| Sval_37 | Polytrichum juniperinum (Mo) | 363/367(99%) | 1/367(0%) | 416.2 | Yes |
| Sval_38 | Polytrichum juniperinum (Mo) | 363/367(99%) | 1/367(0%) | 416.8 | Yes |
| Sval_39 | Saxifraga oppositifolia (An) | 345/346(99%) | 0/346(0%) | 419 | Yes |
| Sval_40 | Saxifraga oppositifolia (An) | 345/345(100%) | 0/345(0%) | 419.5 | Yes |
| Sval_41 | Saxifraga oppositifolia (An) | 343/345(99%) | 0/345(0%) | 419.6 | Yes |
| Sval_42 | Saxifraga oppositifolia (An) | 345/347(99%) | 1/347(0%) | 419.8 | Yes |
| Sval_43 | Saxifraga oppositifolia (An) | 345/345(100%) | 0/345(0%) | 420.9 | Yes |
| Sval_44 | Saxifraga oppositifolia (An) | 345/346(99%) | 0/346(0%) | 420.9 | Yes |
| Sval_45 | Saxifraga oppositifolia (An) | 345/346(99%) | 0/346(0%) | 421.3 | Yes |
| Sval_46 | Lactarius luculentus var. laetus (Mu) | 404/417(97%) | 9/417(2%) | 448.7 | Yes |
Discussion

Detailed and accurate information on forage preference in accordance with seasonal and regional characteristics are very important to understand energy requirement of Svalbard reindeer (*R. tarandus platyrhynchus*) as related to food quality and food quantity [4,7,30–33]. Our LH-PCR approach for fecal dietary analysis from feces could detect several preferred food species through comparison with a constructed LH database and sequencing results. This strategy would increase resolving power in identification and efficiently detect various food sources from collected fecal samples.

Svalbard reindeer are known as selective feeders, preferring lichens over grasses, and preferring grasses over mosses [9]. Our results of the LH-PCR supported the results of the previous study that lichen species may be one of important and preferable food sources in the study sites in Svalbard, especially during the summer season. More than 20% of the total peak area represented lichen species in 2012. In the 2011 samples, lichen species also contributed the highest percentage of the represented 7.4% of total diet composition although the peaks species in 2012. In the 2011 samples, lichen species also season. More than 20% of the total peak area represented lichen sources in the study sites in Svalbard, especially during the summer season. They may be one of important and preferable food species in the composition of the vegetation available in the different regions and annual season.

Terrestrial mammals may make use of intertidal zones as forage sites during resource-restricted periods [34]. Hansen and Aanes [35] reported that kelp or seaweed were foraged by Svalbard reindeer (*R. tarandus platyrhynchus*) under extreme weather conditions such as icing associated with heavy rain-on-snow events. Most of these studies relied on direct observations of feeding behavior [34,35]. We suggest that LH-PCR analysis of fecal samples can be useful for studying foraging strategy in the winter season. In fact, we obtained 112 sequences deposited in GenBank from 4 kelp species and 7 seaweed species growing in the Svalbard or polar regions [36–39]. We then predicted amplification lengths from these sequences on the basis of a calculated relationship between predicted and measured amplification lengths by using the information of the LH database constructed in this study.

Table 2. Cont.

| Sample ID | BLAST results | Amplification length (bp) | Occurrence of identified taxon on Svalbard | Origin of sequence |
|-----------|----------------|--------------------------|------------------------------------------|-------------------|
|           | Description | Identities | Gaps |                                      |                   |
| Sval_47   | Lactarius luculentus var. laetus (Mu) | 406/417(97%) | 9/417(2%) | 448.8 | Yes |
| Sval_48   | Russula laccata (Mu) | 409/409(100%) | 0/409(0%) | 449.1 | Yes |
| Sval_49   | Russula laccata (Mu) | 407/409(99%) | 0/409(0%) | 450.3 | Yes |

Non-food sources

| Sample ID | BLAST results | Amplification length (bp) | Occurrence of identified taxon on Svalbard | Origin of sequence |
|-----------|----------------|--------------------------|------------------------------------------|-------------------|
|           | Description | Identities | Gaps |                                      |                   |
| Sval_50   | Ericoid mycorrhizal sp. (Fu) | 249/285(87%) | 4/285(1%) | 329.8 |                     |
| Sval_51   | Cladosporium sp. (Fu) | 296/296(100%) | 0/296(0%) | 333.5 | Feces |
| Sval_52   | Thelebolus microsporus (Fu) | 295/295(100%) | 0/295(0%) | 334.6 | Feces |
| Sval_53   | Sporormiella vexans (Fu) | 257/257(100%) | 0/257(0%) | 336.6 |                     |
| Sval_54   | Friedmanniomyces endolithicus (Fu) | 268/306(88%) | 13/306(4%) | 339.8 | Feces |
| Sval_55   | Friedmanniomyces endolithicus (Fu) | 268/307(87%) | 11/307(3%) | 340.2 | Feces |
| Sval_56   | Friedmanniomyces endolithicus (Fu) | 268/307(87%) | 11/307(3%) | 340.8 | Feces |
| Sval_57   | Tomentella bryophila (Fu) | 356/368(97%) | 0/368(0%) | 406.3 |                     |

They were arranged by LH length. “Yes” indicates species found in Svalbard. Sequences obtained from feces by cloning represented as “Feces”. Each abbreviation represented as follows: An: angiosperms; Fu: other fungi, which do not form food sources like mushrooms and lichens; Li: lichens; Mo: mosses; Mu: mushroom-forming fungi.

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Table 3. Number of LH peaks in LH profiles and estimated of diet composition on different sampling regions in between 2011 and 2012 (Lovénbreen, Ny-Ålesund in 2011 vs. Brøggerbreen, Ny-Ålesund in 2012 vs. Lovénbreen, Ny-Ålesund in 2012).

| Sampling site          | Number of samples | Number of LH peaks in profiles | Diet composition (relative peak area, %) |
|------------------------|-------------------|--------------------------------|----------------------------------------|
|                        | average | min  | max  | Lichens | Mushrooms | Angiosperms | Other fungi | Unclassified |
| Lovénbreen, Ny-Ålesund in 2011 | 3       | 36.7±4.5 | 32   | 42   | 7.4±1.9 | 1.6±0.8 | 59.8±8.0 | 5.2±3.5 | 2.3±1.5 |
| Brøggerbreen, Ny-Ålesund in 2012 | 3       | 24.3±17.2 | 9    | 43   | 20.8±7.0 | 0.5±0.8 | -       | 36.0±13.6 | 20.0±10.0 |
| Lovénbreen, Ny-Ålesund in 2012 | 9       | 10.7±4.2 | 4    | 19   | 26.0±9.2 | -       | -       | 56.7±17.8 | 8.0±13.8 |

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This *in silico* analysis indicated that LH amplification from kelp and seaweed should result in DNA fragments with lengths in the range of 182.7 to 364.1 bp, which is an appropriate range for LH-PCR analysis.

We considered that the ITS gene may be appropriate marker for dietary analysis of reindeer. Reindeer preferred lichens, mushrooms, and mosses as well as various other plant species as food sources. To detect all potential food sources from feces using LH-PCR, target genes must have enough resolution for various potential food sources and enough length variations. The ITS gene of nuclear ribosomal DNA is commonly sequenced in fungi than any other region of DNA and recommended as representatives of fungal DNA barcode [40,41]. Additionally, Chen and his colleague tested that ITS2 region represents the most suitable region for DNA barcoding applications compared to seven candidate DNA barcode [42]. They tested the discrimination ability of ITS2 in more than 6600 plant samples and identification success rate was recorded 92.7% at the species level although this study was conducted on medicinal plants [42]. Also, It is well known that the ITS gene shows highly length variation compared to other conservative regions [43].

To better interpret forage preferences from fecal samples by using LH profiles, several limitations should be considered. First, it is necessary to construct an additional LH database for food sources used by reindeer not included in this study. Especially the construction of a local LH database would be useful to increase the resolution of the LH-PCR analysis. However, despite these efforts, it remains difficult to identify a food source reliably if the difference in LH length is small between foraged species (Table 1). Therefore, we recommend to apply the LH-PCR analysis in parallel with vegetation surveys on the study area to facilitate a more complete dietary analysis. Second, for technical reasons, the number of PCR cycles or template DNA concentration could affect the results of the LH-profile analysis because of kinetic bias during PCR amplification. In addition, lower DNA concentration in template can reduce detection rate of foraged diet components [44]. However, it has been proposed that decrease of PCR cycles could reduce kinetic bias in the PCR and give highly reproducible data [23,26]. Based on our preliminary experiments, we decided 30 cycles as an appropriate number of cycles for analyzing the fecal samples. Additionally, in this study, we could not compare the change of LH-PCR pattern according to the template DNA concentration because DNA concentration extracted from feces showed lower than other organisms, in general. In further study, obtaining high template DNA would help to increase detection rate and reproducibility. Third, collection of fresh or recently excreted fecal samples may be helpful to increase prey DNA detection success. Decrease in prey detection success were observed when feces were exposed to rain and ultra violet (UV) radiation [12,45]. DNA from feces exposed for long time to environmental conditions were more degraded by various reasons, such as environmental factors (rain and ultra violet (UV) radiation), enzymatic activities, consumed by microbe and so on [12,46]. In this study, 15 feces were successfully amplified among the 18 fecal samples collected. When we collected fecal samples, all fecal samples were collected without selection by time after
excretion. Consideration of time after defecation will increase successful recover rate of prey DNA from feces. Finally, combination with specific blocking primers will selectively prevent amplification of DNA from fungal species that are not reindeer food sources in further study. Such an application of blocking primers will increase the resolution and the accuracy of analyses for discriminating preferred food sources.

Most previous studies involving dietary analysis require direct handling of animals and result in difficulties in the identification of food sources from remains. Our results show that application of LH-PCR analysis would complement the methodological limitations of the traditional approaches. Although there are several limitations to this approach as mentioned above, LH-PCR could be complemented by expanding an LH database for potential food sources and increasing resolving power in identification. We believe that the use of LH-PCR analysis would be an ethical and efficient monitoring tool for investigating the foraging strategies of Svalbard reindeer.

**Author Contributions**

Conceived and designed the experiments: SJ DH SP. Performed the experiments: SJ. Analyzed the data: SJ. Contributed reagents/materials/analysis tools: SJ EJL. Wrote the paper: SJ SP.

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