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Diversity in sea buckthorn (Hippophae rhamnoides L.) accessions with different origins based on morphological characteristics, oil traits, and microsatellite markers

--Manuscript Draft--

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Full Title:	Diversity in sea buckthorn (Hippophae rhamnoides L.) accessions with different origins based on morphological characteristics, oil traits, and microsatellite markers
Short Title:	Diversity in sea buckthorn accessions based on morphological characteristics, oil traits, and SSR markers
Corresponding Author:	Chengjiang Ruan Dalian Medical University Dalian, CHINA
Keywords:	diversity analysis; morphological characteristics; SSR markers; biochemical traits; fatty acid composition
Abstract:	Sea buckthorn (Hippophae rhamnoides) is an ecologically and economically important species. Here, we assessed the diversity of 78 accessions cultivated in northern China using 8 agronomic characteristics, oil traits (including oil content and fatty acid composition) in seeds and fruit pulp, and SSR markers at 23 loci. The 78 accessions included 52 from ssp. mongolica , 6 from ssp. sinensis , and 20 hybrids. To assess the phenotypic diversity of these accessions, 8 agronomic fruit traits were recorded and analyzed using principal component analysis (PCA). The first two PCs accounted for approximately 78% of the variation among accessions. The oil contents were higher in pulp (3.46-38.56%) than in seeds (3.88-8.82%), especially in ssp. mongolica accessions. The polyunsaturated fatty acid (PUFA) ratio was slightly lower in the seed oil of hybrids (76.06%) than that of in ssp. mongolica (77.66%) and higher than that of in ssp. sinensis (72.22%). The monounsaturated fatty acid (MUFA) ratio in the pulp oil of ssp. sinensis (57.00%) was highest, and that in ssp. mongolica (51.00%) was approximately equal to the ratio in the hybrids (51.20%). Using canonical correspondence analysis (CCA), we examined the correlation between agronomic traits and oil characteristics in pulp and seeds. Oil traits in pulp from different origins were correlated with morphological groupings (r = 0.8725, p = 0.0000). To assess the genotypic diversity, 23 SSR markers (including 17 loci previously reported) were used among the 78 accessions with 59 polymorphic amplified fragments obtained and an average PIC value of 0.2845. All accessions were classified into two groups based on the UPGMA method. The accessions of ssp. sinensis and ssp. mongolica were genetically distant. The hybrid accessions were close to ssp. mongolica accessions. The 8 agronomic traits, oil characteristics in seed and pulp oils, and 23 SSR markers successfully distinguished the 78 accessions. These results will be valuable for cultivar identification and genetic diversity analysis in cultivated sea buckthorn.
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	Li Wang
	Xingjun Tian
Response to Reviewers:	General Comments: The manuscript has a lot of issues with the language. Many such sentences have been highlighted in the document attached. Some of these appear as two half statements fused. At other places, the sentences lack clarity. The authors need to re-frame all such statements. Response: Thank you for the valuable suggestion. The sentences mentioned above are all re-framed with clarity. And the revised manuscript was professionally edited by

American Journal Experts (AJE, ID: HS1GCXH7) for the improvement in English quality.

The Materials & Methods needs to be revised at places (please see suggestions). Most trait measures in various tables lack the unit of measurements. Please incorporate that.
Response: Thank you for the valuable suggestion. The places mentioned above in the Materials & Methods have been revised according to the reviewers' suggestions. And the unit of measurements has been added in the tables of the revised manuscript.

Specific comments:
1.The text contradictorily mentions the deployment of 20 SSR primer pairs at certain places (line 357, 450, Header of Table 5) and at other places (Line 27, 41, 46, 197, 358, 373, 383, 451, 482) the use of 23 SSR primer pairs has been mentioned. The supplementary table (S4) gives sequence information for 23, while its header says 20 SSR primers. Table 5, gives information for 23 markers although the Header says 20. Please ensure that all these ambiguities are taken care of.
Response: Twenty-three SSR primer pairs were used in this study. The number '20' at certain places (line 357, 450, Headers of Table 5 and S4 Table) have been replaced by 23 in the revised manuscript.

2.Abstract says 69 polymorphic bands, while in results 59 polymorphic bands are mentioned. This ambiguity also needs to be addressed.
Response: The number of polymorphic bands is 59. It has been revised in the abstract of the revised manuscript.

Introduction:
1. Line 62: 'Two important parameters in……..oil quantity are oil content'. Oil content cannot be a parameter of oil quality. So, this statement needs modification.
Response: The authors agreed with this opinion. The oil content is a parameter of oil yield. The sentence has been revised as bellow.
'Two important parameters in analyzing oil yield and quality are oil content and fatty acid (FA) composition (referred to here as 'oil traits' for simplicity).'

2. Line 78: 'Due to small berries…….artificial hybridization for elite accessions.' The statement needs to be reframed.
Response: Thank you for the valuable suggestion. The statement is reframed as bellow.
'Due to the small berries and thorns of native cultivars (ssp. sinensis), which result in little economic value, the breeding of sea buckthorn has undergone different stages of development in China, such as introduction, domestication, seedling selection and artificial hybridization for elite accessions.'

3.Line 98: 'The diversity analysis…….germplasm'. The authors appear to have fused to incomplete sentences. This needs to be re-written.
All other such statements have been highlighted in the document attached.
Response: Thank you for the valuable suggestion. All such statements are re-written in the revised manuscript.

Materials & Methods
1.Line 139: 'There were three biological replicates……measurement'.
Do the authors mean that 300 berries were taken for the analysis? 100 berries from 2-5 plants/ accession is a good enough number for the analysis.
Response: Yes. 300 berries were taken for the analysis. We collected more than 300 berries per accession and the analyses of other nutrients were performed in our research work, e.g. vitamin C, vitamin E and carotenoids.

2.Line142: '…with over 20 measurements…for each'
This is not clear. Do the authors mean 20 berries per accession?? And how many plants did these berries belong to?
Response: The authors agreed with this view. It means averaged 20 determinations were done for each character. These berries were selected from the berry samples randomly collected from 2-5 ramet plants per accession. This sentence mentioned above has been re-framed for clarity in the revised manuscript as bellow.
'The transverse and longitudinal diameters of berries (BTD and BLD) and the length,

width and thickness of seeds (SL, SW and ST) were measured over 20 times each (on average) by micrometer calipers.'
3.For the oil extraction, were the seeds and fruit pulp weighed prior to oil extraction to maintain some uniformity. This has not been mentioned in the M&M.
The oil contents in both seeds and fruit pulp as mentioned in Line 153 is expressed as percentage. Percentage of what? Seed/pulp weight? The authors need to clearly mention that in the M&M.
In the results (Line 278), the authors mention '….highest oil content (24.68%) based on dry weight.' This means that the weight of the pulp/seed was considered. But, this has not been clearly mentioned either in the M&M or in the Table 3. The units for oil characteristic (min and max) have not been mentioned in the table.
Reponse: Thank you for the valuable suggestion. The method of lipid extraction was described by Yang and Kallio (2001). Samples (1 g) of seeds and fruit pulp were isolated from freeze-dried berries and lipids from the samples were extracted with chloroform/methanol (2:1, v/v) with mechanical homogenization of the tissues. The oil contents (percentages) in seeds and fruit pulp were calculated (oil % in seeds and lyophilized fruit pulp). The fatty acid composition was also expressed as a weight percentage of the total fatty acids. The units (weight percentages) for oil characteristics in Table 3 and Table 4 have been added in the revised manuscript.

4.Line 197: 'Twenty-three polymorphic microsatellite loci (SSR) developed using RNA-Seq was evaluated and loci SB1-SB17 were previously reported'.
Please mention here the names of the SSR markers (SB1-SB23). Nowhere in the text have they been mentioned except for tables. Then it can be mentioned that SB1-17 were previously deployed (Ref. 14).
The authors need to clearly mention in the introduction itself that in a previous study, RNA seq analysis was done to generate SSR markers and these were tested on 31 accessions. The 17 SSR markers developed in that study have been utilized in the present endeavor for genetic diversity assessment of larger set of accessions. This description in the 'introduction' will bring more clarity in the text. This previous study and its outcomes should be mentioned clearly in the 'Introduction' so that its extension in the present study can be deciphered.
Reponse: Thank you for the valuable suggestion. The sentences mentioned above have been re-framed for clarity in the revised manuscript as bellow.
'Twenty-three polymorphic microsatellite loci (SB1-SB23) developed using RNA-Seq were evaluated. Of these, 17 (SB1-SB17) had been deployed in a previous study by the group [14].'
And the authors added the statements of 17 RNA-Seq SSR markers developed in our previous study and mentioned these SSR markers have been utilized in the present endeavor for genetic diversity assessment of larger set of accessions in the revised manuscript.
'In our previous study, 17 RNA-Seq SSR markers (SB1-SB17) were developed and validated on 31 accessions, which were utilized in the present study for genetic diversity assessment of larger set of accessions [14].'

Results
1. Line 246: 'In previous mutilocation trials in Suiling (47°14′N, 127°06′E; 202 m) and Dengkou (40°43′N, 106°30′E; 1053m, Inner Mongolia), the fruit characteristics of 11 large……'.
How many berries per accession were taken for this analysis? The data should be represented as + SD in Table S7.
Reponse: 300 berries of each cultivar were randomly sampled and divided into 3 groups (100 berries were divided into 1 group) to determine the hundred berry weight (HBW). 20 berries of each cultivar were randomly sampled to determine the transverse, longitudinal diameters of berries and berry shape indices (BTD, BLD and BSI). The data has been represented as + SD in S7 Table in the revised manuscript.

2. Line 302: 'Small variations were found in the proportion of linoleic acid in seed oil (40.44 – 42.87%). Its proportion in hybrids were slightly higher than in ssp. mongolica (42.87% vs 42.10%.....'
Are these differences significant?
Reponse: These differences are significant despite small variations. The content of seed oil in hybrids is lower than that in ssp. mongolica. However, the proportion of linoleic acid (an important polyunsaturated fatty acid) in seed oil is higher in hybrids

	than that in ssp. mongolica, which showed high oil quality of seed oil in hybrids. 3. Table 4: How is the oil content being measured? Total oil per gram weight of seeds and pulp or some other measure? Reponse: The method of lipid extraction was described by Yang and Kallio (2001). Samples (1 g) of seeds and fruit pulp were isolated from freeze-dried berries and lipids from the samples were extracted with chloroform/methanol (2:1, v/v) with mechanical homogenization of the tissues. The oil contents (percentages) in seeds and fruit pulp were calculated (oil % in seeds and lyophilized fruit pulp). Tables and Figures 1. Table 1: Since the authors have already mentioned that 2-5 ramet plants were collected per accession. The columns indicating the number of plants taken per accession can be removed from the Table. Reponse: The columns indicating the number of plants taken per accession have been removed from Table 1 in the revised manuscript. 2. Table 3: The units for the min. and max values of the oil characteristics have not been mentioned in the table. Similarly mention the units of measurement for each of the component in Table 4. Reponse: The units of the oil characteristics have been added in the headers of Table 3 and Table 4. 3. Table 3 & 4: The different fatty acid names should be included in the first column. Example: Oleic (18:1), Palmitic acid (16:0) etc. Reponse: Thank you for the valuable suggestion. The different fatty acid names are included in the first column of Table 3 and Table 4 according to the examples. 4. Table S1: This table again classifies all the lines used as 'cultivars'. Are these accessions or cultivars? Please check. Reponse: The 'accession' has replaced the 'cultivar' in S1 Table of revised manuscript. 5. Table S3 carries a different header than the one that has been listed at the end of the manuscript. Please change that. Reponse: The header of S3 Table listed at the end of the manuscript has been changed in the revised manuscript. 6. Table S7: The header for this table has been titled as Table S5. Please correct. Also it mentions 'two experimental fields' although it has data from three places. So, please correct. Reponse: The header for S7 Table has been corrected. The mutilocation trials were performed in two experimental fields (Suiling and Dengkou). Russia is the country of origin of those cultivars and the related data were provided by the units where they were introduced.
Additional Information:	
Question	**Response**
Financial Disclosure Enter a financial disclosure statement that describes the sources of funding for the work included in this submission. Review the submission guidelines for detailed requirements. View published research articles from *PLOS ONE* for specific examples. This statement is required for submission and **will appear in the published article** if the submission is accepted. Please make	This research was financially supported by the Natural Science Foundation of China (NSFC)(Grant No. 31100489), which was received by He Li. https://isisn.nsfc.gov.cn/egrantweb/

sure it is accurate. **Unfunded studies** Enter: *The author(s) received no specific funding for this work.* **Funded studies** Enter a statement with the following details: • Initials of the authors who received each award • Grant numbers awarded to each author • The full name of each funder • URL of each funder website • Did the sponsors or funders play any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript? • **NO** - Include this sentence at the end of your statement: *The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.* • **YES** - Specify the role(s) played. * typeset	
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Ethics Statement Enter an ethics statement for this submission. This statement is required if the study involved: • Human participants • Human specimens or tissue • Vertebrate animals or cephalopods • Vertebrate embryos or tissues • Field research Write "N/A" if the submission does not require an ethics statement. General guidance is provided below. Consult the submission guidelines for detailed instructions. **Make sure that all information entered here is included in the Methods section of the manuscript.**	N/A

Format for specific study types **Human Subject Research (involving human participants and/or tissue)** • Give the name of the institutional review board or ethics committee that approved the study • Include the approval number and/or a statement indicating approval of this research • Indicate the form of consent obtained (written/oral) or the reason that consent was not obtained (e.g. the data were analyzed anonymously) **Animal Research (involving vertebrate animals, embryos or tissues)** • Provide the name of the Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board that reviewed the study protocol, and indicate whether they approved this research or granted a formal waiver of ethical approval • Include an approval number if one was obtained • If the study involved *non-human primates*, add *additional details* about animal welfare and steps taken to ameliorate suffering • If anesthesia, euthanasia, or any kind of animal sacrifice is part of the study, include briefly which substances and/or methods were applied **Field Research** Include the following details if this study involves the collection of plant, animal, or other materials from a natural setting: • Field permit number • Name of the institution or relevant body that granted permission	
Data Availability Authors are required to make all data underlying the findings described fully available, without restriction, and from the time of publication. PLOS allows rare exceptions to address legal and ethical concerns. See the PLOS Data Policy and FAQ for detailed information.	Yes - all data are fully available without restriction

A Data Availability Statement describing where the data can be found is required at submission. Your answers to this question constitute the Data Availability Statement and **will be published in the article**, if accepted. **Important:** Stating 'data available on request from the author' is not sufficient. If your data are only available upon request, select 'No' for the first question and explain your exceptional situation in the text box. Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction?	
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Additional data availability information:	

Diversity in sea buckthorn (*Hippophae rhamnoides* L.) accessions with different origins based on morphological characteristics, oil traits, and microsatellite markers

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Abstract

Sea buckthorn (*Hippophae rhamnoides*) is an ecologically and economically important species. Here, we assessed the diversity of 78 accessions cultivated in northern China using 8 agronomic characteristics, oil traits (including oil content and fatty acid composition) in seeds and fruit pulp, and SSR markers at 23 loci. The 78 accessions included 52 from ssp. *mongolica*, 6 from ssp. *sinensis*, and 20 hybrids. To assess the phenotypic diversity of these accessions, 8 agronomic fruit traits were recorded and analyzed using principal component analysis (PCA). The first two PCs accounted for approximately 78% of the variation among accessions. The oil contents were higher in pulp (3.46-38.56%) than in seeds (3.88-8.82%), especially in ssp. *mongolica* accessions. The polyunsaturated fatty acid (PUFA) ratio was slightly lower in the seed oil of hybrids (76.06%) than that of in ssp. *mongolica* (77.66%) and higher than that of in ssp. *sinensis* (72.22%). The monounsaturated fatty acid (MUFA) ratio in the pulp oil of ssp. *sinensis* (57.00%) was highest, and that in ssp. *mongolica* (51.00%) was ~~approximately~~ equal to the ratio in the hybrids (51.20%). Using canonical correspondence analysis (CCA), we examined the correlation between agronomic traits and oil characteristics in pulp and seeds. Oil traits in pulp from different origins were correlated with morphological groupings (r = 0.8725, p = 0.0000). To assess the genotypic diversity, 23 SSR markers (including 17 loci previously reported) were used among the 78 accessions with 59 polymorphic amplified fragments obtained and an average PIC value of 0.2845. All accessions were classified into two groups based on the UPGMA method. The accessions of ssp. *sinensis* and ssp. *mongolica* were genetically distant. The hybrid

accessions were close to ssp. *mongolica* accessions. The 8 agronomic traits, oil characteristics in seed and pulp oils, and 23 SSR markers successfully distinguished the 78 accessions. These results will be valuable for cultivar identification and genetic diversity analysis in cultivated sea buckthorn.

Introduction

Sea buckthorn (*Hippophae rhamnoides* L.) is a hardy winter shrub that is naturally distributed throughout Asia and Europe. It is an economically valuable species, divided into eight subspecies. Of these subspecies, ssp. *sinensis* and *mongolica* are mainly distributed in Asia, where they are abundant and commercially cultivated [1−2]. The fruits of sea buckthorn are rich in a variety of phytochemicals with physiological properties, such as lipids, carotenoids, ascorbic acid, tocopherols, and flavonoids [3−5]. The main applications for the fruits include food, cosmetics, and pharmaceutical products [6−7]. One of the most requested products for therapeutic practices is sea buckthorn oil, which is extracted from both seeds and fruit pulp. The applications of sea buckthorn oil include healing of the skin, mucosa, and immune systems, especially in cancer and cardiovascular disease therapy [8−9].

Two important parameters in analyzing oil yield and quality are oil content and fatty acid (FA) composition (referred to here as 'oil traits' for simplicity). Sea buckthorn seed and pulp oils are considered the most valuable products of the berries with a unique FA composition [10]. The seed oil contains omega-3 (α-linolenic acid) and omega-6 (linoleic acid) FAs, and the pulp oil is characterized by a high concentration of FAs

from the omega-7 group (e.g., palmitoleic acid). Seed oil is rich in unsaturated FAs (commonly 30-40% linoleic acid and 20-35% linolenic acid) [10]. The soft parts (pulp and peel) of the berries have an FA composition that differs from the seeds that is characterized by a high level of palmitoleic acid (16–54%), which is very uncommon in plants. The oil traits of sea buckthorn berries vary greatly according to their origin, based on the climatic and geological conditions of the growing areas [11].

Sea buckthorn adapts well to extreme conditions, including drought, salinity, alkalinity, and extreme temperatures [12]. Its vigorous vegetative reproduction and strong, complex root system with nitrogen-fixing nodules make it an optimal pioneer plant for soil and water conservation. For these reasons, sea buckthorn is cultivated widely in arid and semiarid areas of China [13]. ~~Due to the small berries and thorns of native cultivars (ssp. *sinensis*), which result in little economic value,~~ ~~the~~ breeding of sea buckthorn has undergone different stages of development in China, such as introduction, domestication, seedling selection and artificial hybridization for elite accessions. The cultivars of ssp. *mongolica* (introduced from Russia and Mongolia), ssp. *sinensis* (China origin) and hybrids (ssp. *mongolica* × ssp. *sinensis*) are abundant in northern China [14]. However, as a perennial woody plant, traditional cross breeding that takes ~~a~~ long time and has low efficiency cannot meet the needs of modern production in sea buckthorn. It is essential for economic production to utilize molecular marker-assisted breeding (MAB) in sea buckthorn, especially to breed accessions ~~associated~~ with desirable oil traits. An essential step in this process is the genetic analysis of sea buckthorn germplasm. At present, molecular markers are mainly used

for the analysis of genetic diversity, the taxonomic and geographic origin of cultivars, sex determination and population genetic structure in sea buckthorn [14−16]. SSR (simple sequence repeat, microsatellite) markers, with 1- to 6-bp DNA regions repeated in tandem, have been used in these analyses for their advantages of codominance, random distribution throughout the genome, easy detection, and high polymorphism and reproducibility [17]. Currently, an increasing number of microsatellite markers are being developed in sea buckthorn using high-throughput sequencing techniques for transcriptome datasets (RNA-Seq), which have become valuable resources for SSR discovery [14, 18]. In our previous study, 17 RNA-Seq SSR markers (SB1-SB17) were developed and validated on 31 accessions, which were utilized in the present study for genetic diversity assessment of larger set of accessions [14].

Diversity analysis helps clarify the relationships between germplasm characteristics and genotype and will improve our understanding of sea buckthorn germplasm to achieve greater production with higher quality regarding the important traits correlated with germplasm [19].

In the present study, 78 accessions of sea buckthorn with variation in fruit traits were selected as materials. The aim of this study is to report the phenotypic characteristics and oil traits in fruit pulp and seeds and the genetic diversity of the 78 sea buckthorn accessions in northern China, providing a foundation for MAB in sea buckthorn.

Materials and methods

Plant materials

Berries and leaves of 78 sea buckthorn accessions belonging to ssp. *mongolica* (52 accessions), ssp. *sinensis* (6 accessions) and hybrids (ssp. *mongolica* × ssp. *sinensis*, 20 accessions) were collected from the end of July to mid-September in 2015. Table 1 summarizes information on the plant materials. Three research institutes located in northern China, the Institute of Selection and Breeding of *Hippophae* (42°26′N, 121°28′E; 380 m) in Fuxin, the Research Institute of Berry (47°14′N, 127°06′E; 202 m) in Suiling and the Jiuchenggong Breeding Base of Sea Buckthorn (39°40′N, 110°09′E; 1400 m) in Dongsheng, provided 76 accessions of sea buckthorn samples (Fig 1, S1 Table). The other two accessions, Quyisike and Zhongguoshaji^{wild}, were harvested from cultivated fields in Qinghe (46°40′N, 90°22′E; 1218 m) and Datong (36°53′N, 101°35′E; 2800 m) (Fig 1, S1 Table, S2 Table). These areas have various geographical and climatic conditions (S3 Table).

The young leaves of each plant were kept at −80 °C for use. The berries of each accession were pooled and frozen as quickly as possible at −20 °C. When all plant materials were harvested, the berries were transferred to –50 °C for storage until analysis.

Fig 1. The 78 sea buckthorn accessions from five cultivated lands used in this study.

Table 1. Accessions of sea buckthorn used for the study.

No.	Accession name	Abbrev.[a]	Collection site	ssp.[b]	No.	Accession name	Abbrev.[a]	Collection site	ssp.[b]

1	Zhuangyuanhuang	ZYH	Fuxin	M	40	E13-10	E13-10	Suiling	M
2	Wucifeng	WCF	Fuxin	M	41	E13-11	E13-11	Suiling	M
3	Liusha-1	LS1	Fuxin	M	42	E13-14	E13-14	Suiling	M
4	Siberia rumianes	SR	Fuxin	M	43	HS-1	HS1	Suiling	M
5	Fangxiang	FX	Fuxin	M	44	HS-4	HS4	Suiling	M
6	Yalishanda-12	YLSD12	Fuxin	M	45	HS-9	HS9	Suiling	M
7	Jiuyuehuang	JYH	Fuxin	M	46	HS-10	HS10	Suiling	M
8	Nanren	NR	Fuxin	M	47	HS-12	HS12	Suiling	M
9	Botanical garden	BG	Fuxin	M	48	HS-14	HS14	Suiling	M
10	Zajiao-1	ZJ1	Fuxin	H	49	HS-18	HS18	Suiling	M
11	Zajiao-2	ZJ2	Fuxin	H	50	HS-20	HS20	Suiling	M
12	Zajiao-3	ZJ3	Fuxin	H	51	HS-22	HS22	Suiling	M
13	MZ-14	MZ14	Suiling	M	52	Xin’e-1	XE1	Suiling	M
14	Shoudu	SD	Suiling	M	53	Xin’e-2	XE2	Suiling	M
15	Fenlan	FL	Suiling	M	54	Xin’e-3	XE3	Suiling	M
16	Aertai	AET	Suiling	M	55	Zhongguoshaji	ZGSJ	Suiling	S
17	Chengse	CS	Suiling	M	56	EZ-4	EZ4	Suiling	H
18	Chuyi	CY	Suiling	M	57	Za-56	Za56	Suiling	H
19	Hunjin	HJ	Suiling	M	58	Za1-2	Za1-2	Suiling	H
20	Jinse	JS	Suiling	M	59	Za05-6	Za05-6	Suiling	H
21	Juren	JR	Suiling	M	60	Za05-20	Za05-20	Suiling	H
22	Xiangyang	XY	Suiling	M	61	Za05-21	Za05-21	Suiling	H
23	Yousheng	YS	Suiling	M	62	Za4	Za4	Suiling	H
24	Katuni	KTN	Suiling	M	63	Za13-19	Za13-19	Suiling	H
25	Wulangemu	WLGM	Suiling	M	64	Za13-25	Za13-25	Suiling	H
26	TF1	TF1	Suiling	M	65	Juda	JD	Dongsheng	S
27	TF2-13	TF2-13	Suiling	M	66	Jianpingdahuang	JPDH	Dongsheng	S
28	TF2-23	TF2-23	Suiling	M	67	Manhanci	MHC	Dongsheng	S
29	TF2-24	TF2-24	Suiling	M	68	Zhongxiongyou	ZXY	Dongsheng	S
30	TF2-36	TF2- 36	Suiling	M	69	Liaofuza	LFZ	Dongsheng	H

31	Suiji-1	SJ1	Suiling	M	70	Zaciyou-1	ZCY1	Dongsheng	H
32	Suiji-3	SJ3	Suiling	M	71	Zaciyou-10	ZCY10	Dongsheng	H
33	Suiji-4	SJ4	Suiling	M	72	Zaciyou-12	ZCY12	Dongsheng	H
34	HD-3	HD3	Suiling	M	73	Xinzaci-26	XZC26	Dongsheng	H
35	E10-06	E10-06	Suiling	M	74	Shiciyou-2	SCY2	Dongsheng	H
36	E10-34	E10-34	Suiling	M	75	Shiciyou-5	SCY5	Dongsheng	H
37	E10-42	E10-42	Suiling	M	76	Shiciyou-30	SCY30	Dongsheng	H
38	E10-47	E10-47	Suiling	M	77	Zhongguoshaji*wild*	ZGSJ*wild*	Datong	S
39	E13-00	E13-00	Suiling	M	78	Qiuyisike	QYSK	Qinghe	M

[a] Abbrev., abbreviation.

[b] ssp., subspecies; M, ssp. *mongolica*; S, ssp. *sinensis*; H, hybrid (ssp. *mongolica* ♀ × ssp. *sinensis* ♂).

Morphological characteristics of fruit

Hundred berry weight (HBW) was the weight of 100 fresh berries after they were picked from bushes. Hundred seed weight (HSW) was the weight of 100 seeds after air drying at room temperature (25 °C) for 2 weeks [20]. There were three biological replicates for each measurement. The transverse and longitudinal diameters of berries (BTD and BLD) and the length, width and thickness of seeds (SL, SW and ST) were measured over 20 times each (on average) by micrometer calipers. The berry shape indices (BSIs) were estimated by the ratio of BLD to BTD. The minimum (Min), maximum (Max), mean ± standard deviation (SD), and coefficient of variation (CV%) were reported.

Oil extraction and FA analysis in seeds and pulp

148 The methods of lipid extraction, transesterification (methylation) and purification of
149 methyl esters of the lipid extracts were described by Yang and Kallio [11]. Briefly,
150 samples (1 g) of seeds and fruit pulp were isolated from freeze-dried berries and lipids
151 from the samples were extracted with chloroform/methanol (2:1, v/v) with mechanical
152 homogenization of the tissues. The purified oils were filtered before the solvent was
153 removed on a rotary evaporator. The lipids were weighed, and the oil contents
154 (percentages) in seeds and fruit pulp were calculated. Three biological replicates were
155 taken for analysis. Lipids were stored in chloroform at −20 °C until analysis.

156 The oil (10 mg) was transesterified by sodium methoxide catalysis [11, 21]. It was
157 dissolved in sodium-dried diethyl ether (1 ml) and methyl acetate (20 µl). Then, 1 M
158 sodium methoxide in dry methanol (20 µl) was added, and the solution was agitated
159 briefly and incubated for 5 min at room temperature. The reaction was stopped by
160 adding a saturated solution of oxalic acid in diethyl ether (30 µl) with brief agitation.
161 The mixture was centrifuged at 1500 g for 2 min, and the supernatant was dried in a
162 gentle stream of nitrogen. Fresh hexane (1 ml) was added and the solution was filtered
163 with microporous filtering films (0.22 µm) for analysis.

164 Fatty acid methyl esters (FAMEs) were analyzed with a gas chromatography-
165 tandem mass spectrometry (GC/MS/MS) system (model AxION® iQT™, PerkinElmer,
166 Shelton, CT, USA). Chromatographic separation was achieved using a DB-23 capillary
167 column (60 m × 0.25 mm × 0.25 µm; Agilent Technologies, Santa Clara, CA, USA)
168 with the following temperature program: initial temperature 50 °C, hold for 1 min, heat
169 to 175 °C at 25 °C/min, then heat to 215 °C at 3 °C/min and hold for 10 min, heat to

230 °C at 3 °C/min and hold for 5 min. The inlet was operated in split mode (1:20) at a temperature of 250 °C with helium as the carrier gas at constant flow of 1.0 mL/min. The transfer line temperature was 215 °C, and the MS ion source was set to 230 °C. MS detection was carried out in electron impact (EI) ionization mode, scanning all masses from 45–400 amu. FAME components were identified based on mass spectral comparison with an external standard (Supelco 37 Component FAME Mix, Sigma-Aldrich, St. Louis, MO, USA) and previous studies [10−11]. The main FA composition was expressed as a weight percentage of the total FAs from three replicates. The minimum, maximum, mean ± SD, and CV% were reported.

Statistical analysis

The data analysis for morphological traits and oil characteristics was performed with SPSS® 24.0 (IBM®). The following parameters were evaluated: mean, minimum value, maximum value, SD and CV%. One-way analysis of variance (ANOVA) was used in the comparison of all traits among subsp. of *sinensis*, subsp. of *mongolica* and hybrids. Pearson correlation coefficients were calculated to analyze the relationship between pairs of 8 agronomic traits. Principal component analysis (PCA) was used to determine relationships among the accessions. In addition, a canonical correspondence analysis (CCA) was applied to the data between morphological characteristics and oil traits in different tissues (seeds and pulp).

DNA extraction and SSR analysis

192 Total genomic DNA was extracted from young leaves using the TaKaRa MiniBEST
193 Plant Genomic DNA Extraction Kit (TaKaRa, Beijing, China) based on the
194 manufacturer's protocol. The purity and quantity of extracted DNA were evaluated by
195 gel electrophoresis and a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific,
196 Waltham, MA, USA). Twenty-three polymorphic microsatellite loci (SB1-SB23)
197 developed using RNA-Seq were evaluated. Of these, 17 (SB1-SB17) had been
198 deployed in a previous study by the group [14] (S4 Table). PCR amplification was
199 performed in 20 μl volumes containing 40 ng of DNA template, 1× PCR buffer, 1.5 mM
200 $MgCl_2$, 0.15 mM of each dNTP (Takara, Dalian, China), 1.5 U of Taq polymerase
201 (Takara, Dalian, China) and 0.5 μM of each primer. The PCR conditions included an
202 initial denaturation at 94 °C for 2 min and 35 cycles of 30 s at 94 °C for denaturation,
203 30 s at 54–60 °C for annealing and 45 s at 72 °C for extension, with a final extension
204 for 7 min at 72 °C using a C1000 Touch™ Thermal Cycler (Bio-Rad, Berkeley, CA,
205 USA). PCR products were electrophoresed on 8% nondenaturing polyacrylamide gels
206 using an SE 600 Ruby Standard Dual Cooled Vertical Unit (GE Healthcare Life
207 Sciences, Pittsburgh, PA, USA) and visualized by silver staining.

208 The microsatellites were scored as codominant markers for genetic diversity
209 analysis. The number of alleles (Na), effective number of alleles (Ne), observed and
210 expected heterozygosity (Ho and He), Shannon's information index (Is) and
211 polymorphic information content (PIC) for each of the genic SSR markers were
212 calculated using GenAlEx 6.5 [22−23] and PowerMarker version 3.25 [24] software
213 packages. A genetic similarity matrix based on the proportion of shared alleles was

generated, and a UPGMA tree was constructed using PowerMarker. The dendrogram was displayed using MEGA 6 software [25] to reveal genetic relationships between the 78 sea buckthorn accessions.

Results

Morphological characterization of berries and seeds

Descriptive statistical analysis of 8 agronomic fruit traits for the 78 sea buckthorn accessions is shown in Tables 2, S5 and S6. Relatively high CV values were observed for the HBW, BLD, and HSW (> 20%). The highest CV% was observed for the HBW (39.12%), which varied from 8.52 to 69.74 g. ANOVA ($p < 0.05$) showed that the HBW of ssp. *mongolica* berries was 47.69 ± 11.03 g, which was much higher than those of ssp. *sinensis* berries (10.73 ± 1.54 g) and hybrids (31.44 ± 13.84 g). In hybrids, the HBW values were high in EZ4, Za56, Za1-2, Za05-6 and Za05-21(> 45 g), which were approximately the size of those in ssp. *mongolica* berries on average (S6 Table). The BTD varied from 5.54 to 10.80 mm, and the BLD varied from 4.83 to 14.25 mm. In addition, the BLD of berries from ssp. *mongolica* was higher than the BTD, and this relationship was the opposite in berries of ssp. *sinensis*. According to BSI values, the berry shapes of the three groups were significantly different ($p = 0.000$): oblong berries for ssp. *mongolica* (1.35 ± 0.20), oblate berries for ssp. *sinensis* (0.90 ± 0.05) and circular berries for the hybrids (1.08 ± 0.11). The HSW varied from 0.61 to 2.19 g with an average of 1.45 g. Similar to the HBW, there were significant differences in the HSW among seeds from ssp. *mongolica*, ssp. *sinensis*, and hybrids ($p = 0.000$). The SL

varied from 2.00 to 3.49 mm, and the SW varied from 2.98 to 7.43 mm. The ST varied from 1.54 to 2.73 mm, with an average of 1.93 mm. Overall, the agronomic characteristics of seeds (HSW, SL, SW, and ST) showed relatively low coefficients of variation, ranging from 11.50–24.33%; however, the berries (HBW, BTD, BLD, and BSI) had high CV%s.

Table 2. Fruit traits of sea buckthorn berries of two different subspecies and hybrid accessions[a].

Trait name	**Abbrev.**[b]	**ssp. *mongolica***	**ssp. *sinensis***	**Hybrid**
Hundred berry weight (g)	HBW (g)	47.69 ±11.03a	10.73 ± 1.54c	31.44 ±13.84b
Berry transverse diameter (mm)	BTD (mm)	8.17 ± 0.99a	5.84 ± 0.23b	7.61 ± 1.24a
Berry longitudinal diameter (mm)	BLD (mm)	10.90 ± 1.48a	5.20 ± 0.19c	8.15 ± 1.18b
Berry shape index	BSI	1.35 ± 0.20	0.90 ± 0.05	1.08 ± 0.11
Hundred seed weight (g)	HSW (g)	1.60 ± 0.28a	0.79 ± 0.23c	1.28 ± 0.25b
Seed length (mm)	SL (mm)	5.91 ± 0.68a	3.31 ± 0.27c	4.64 ± 0.56b
Seed width (mm)	SW (mm)	2.76 ± 0.27a	2.18 ± 0.18c	2.52 ± 0.22b
Seed thickness (mm)	ST (mm)	1.98 ±0.18a	1.67 ± 0.16 b	1.86 ± 0.26a

[a] Values with different lowercase letters (a–c) are significantly different at $p < 0.05$.

[b] Abbrev., Abbreviation.

In previous multilocation trials in Suiling (47°14′N, 127°06′E; 202 m) and Dengkou (40°43′N, 106°30′E; 1053 m, Inner Mongolia), the fruit characteristics of 11 large berry accessions (AET, CS, CY, HJ, JS, JR, XY, YS, KTN, WLGM and SJ1) were comparatively analyzed (S7 Table). The HBWs values in Suiling (38.33–67.59 g) were higher than those in Dengkou (32.87–63.85 g). For all the introduced cultivars, the HBW values in the two experimental fields were lower than those in their country of origin, Russia. The phenotypic characteristics of sea buckthorn berries showed differences due to their origins, different parts of fruit analyzed, climatic and growing conditions. In this study, 78 accessions were selected for their good adaptabilities to growth sites.

PCA was performed using fruit characteristics (Fig 2). The first two PCs explained 78.11% of the total morphological variance. The first PC accounted for 41.74% of the variance. It was associated with BTD, HBW, ST, HSW, and SW in descending order. Therefore, these traits were important attributes for the classification of sea buckthorn accessions. The second PC accounted for 36.37%, which were correlated with BSI, SL, and BLD in descending order. The plot shows the distribution of 78 sea buckthorn accessions on PC1 and PC2 (Fig 2). The ssp. *mongol*ica accessions with larger berries tended to cluster together, mainly positive on PC2. Six accessions of ssp. *sinensis* with the smallest berries were negative on both PC1 and PC2. The hybrids were largely distributed between the above two groups. Some hybrids (including ZCY1, ZCY10, ZCY12, XZC26, SCY2, and SCY5) were close to the accessions from ssp. *sinensis*.

Fig 2. Two-dimensional scatter plot for the first two principal components (PC1 and PC2) based on the agronomic fruit characteristics of 78 sea buckthorn accessions. Numbers associated with symbols are the variety codes listed in Table 1. ▲ = ssp. *mongolica*; ● = ssp. *sinensis*; ◇ = hybrid.

Oil characterization in seeds and seedless parts

The oil characteristics of seeds and seedless parts (pulp and peel) among the 78 accessions are summarized in Tables 3 and Table 4. One special feature of sea buckthorn fruit was the high oil content in the pulp and peel (20.41%), in contrast to the oil content in the seeds (8.82%). A higher CV% was observed in pulp oil (42.72%) and varied over a wide range, from 3.46 to 38.56%. The pulp fraction of berries of ssp. *mongolica* had the highest oil content (24.68%) based on dry weight. The lowest pulp oil content (7.10%) on average was found in the berries of ssp. *sinensis*. In hybrids, the berries of ZJ2 contained 27.22% pulp oil, which slightly exceeded that of ssp. *mongolica* on average (S6 Table). The seed oil content varied from 3.88 to 12.75% with an average of 8.82%. The seeds of ssp. *mongolica* had the highest oil contents, with an average of 9.46%, and those of the other two groups did not differ significantly.

Table 3. Oil characteristics of pulp and seeds of 78 sea buckthorn accessions (weight percentages).

Character	Pulp				Seed			
	Min[a]	Max[b]	Mean ± SD[c]	CV[d] (%)	Min[a]	Max[b]	Mean ± SD[c]	CV[d](%)
oil content	3.46	38.56	20.41 ± 8.72	42.72	3.88	12.75	8.82 ± 1.86	21.08

Palmitic acid (16:0)	24.52	53.08	36.26 ± 4.83	13.32	3.84	11.77	6.55 ± 1.39	21.16
Palmitoleic acid (16:1n7)	17.93	57.75	35.12 ± 7.64	21.76	tr[e]	tr[e]	tr[e]	
Stearic acid (18:0)	0.38	5.12	1.26 ± 0.70	55.58	1.41	4.58	2.16 ± 0.43	20.11
Oleic acid (18:1n9)	1.44	23.43	8.72 ± 4.72	54.13	3.05	25.95	13.25 ± 4.04	30.50
Vaccenic acid (18:1n7)	3.51	24.24	7.68 ± 4.09	53.28	0.45	2.38	1.20 ± 0.47	39.17
Linoleic acid (18:2n6)	3.02	17.40	9.97 ± 3.18	31.91	34.22	52.75	42.17 ± 3.60	8.54
α-Linolenic acid (18:3n3)	0.12	7.16	1.00 ± 1.03	102.83	21.37	47.16	34.67 ± 4.42	12.75

288 [a] Minimum value.

289 [b] Maximum value.

290 [c] Standard deviation.

291 [d] Coefficient of variation expressed as a percentage.

292 [e] tr, trace (< 0.5%).

293 **Table 4. Oil content and fatty acid composition in the seeds and fruit pulp of sea buckthorn berries of different origins[a] (weight percentages).**

	Pulp oil			Seed oil		
Character	**ssp. *mongolica***	**ssp. *sinensis***	**Hybrid**	**ssp. *mongolica***	**ssp. *sinensis***	**Hybrid**
oil content	24.68 ± 6.79 a	7.10 ± 3.28c	13.34 ± 4.85b	9.46 ± 1.56a	6.70 ± 1.32b	7.78 ±1.84b
Palmitic acid (16:0)	37.68 ± 4.64a	29.39 ± 3.71b	34.62 ± 3.14a	6.52 ± 1.16	7.41 ± 1.55	6.38 ± 1.82
Palmitoleic acid (16:1n7)	37.43 ±7.09a	23.65 ± 4.16b	32.55 ± 5.84a	tr[b]	tr[b]	tr[b]
Stearic acid (18:0)	1.08 ±0.69b	1.73 ± 0.64a	1.59 ± 0.57ab	2.13 ± 0.29	2.19 ± 0.44	2.23 ± 0.69
Oleic acid (18:1n9)	7.56 ±3.97b	16.67 ± 6.84a	9.33 ± 3.40b	12.62 ± 3.75b	16.37 ± 3.77a	13.96 ± 4.46ab
Vaccenic acid (18:1n7)	6.01 ±1.79c	16.68 ± 6.20a	9.32 ± 3.63b	1.07 ± 0.37b	1.80 ± 0.39a	1.37 ± 0.55b
Linoleic acid (18:2n6)	9.55 ±2.76ab	8.34 ± 5.54b	11.53 ± 2.92a	42.10 ± 3.08	40.44 ± 4.06	42.87 ± 4.62
α-Linolenic acid (18:3n3)	0.69 ±0.41b	3.54 ± 2.09a	1.07 ± 0.64b	35.56 ± 4.13a	31.78 ± 2.91b	33.20 ± 4.89 ab
MUFA	51.00 ±5.38b	57.00 ± 9.46a	51.20 ± 3.52b	13.69 ± 3.93b	18.18 ± 4.09a	15.33 ± 4.90ab
PUFA	10.24 ±2.98	11.89 ± 7.54	12.60 ±3.37	77.66 ± 4.31a	72.22 ±5.54b	76.06 ± 6.23ab

294 [a] Values with different lowercase letters (a–c) are significantly different at $p < 0.05$.

295 [b] tr, trace (< 0.5%).

For sea buckthorn, the FA composition in the seed oil differed significantly from that in the pulp oil. The proportions of FAs ranked from high to low as linoleic (18:2n6), *α*-linolenic (18:3n3), oleic (18:1n9), palmitic (16:0), stearic (18:0) and vaccenic (18:1n7) acids in the seed oil of most accessions (Table 4). Linoleic acid varied from 34.22 to 52.75% with an average of 42.17%. The proportion of *α*-linolenic acid varied from 21.37 to 47.16% with an average of 34.67%. High CV values were observed in oleic (30.50%) and vaccenic (39.17%) acids. Furthermore, the level of palmitoleic acid (16:1n7, < 0.5%) was extremely low in the seed oil. The FA composition of sea buckthorn seeds was similar among berries of the two different subspecies and hybrid accessions. Small variations were found in the proportion of linoleic acid in seed oil (40.44 – 42.87%). Its proportion in hybrids was slightly higher than in ssp. *mongolica* (42.87% vs 42.10%), and had the highest value of the samples from the two different subspecies and hybrid accessions. *α*-Linolenic acid showed little variation, with a larger proportion in ssp. *mongolica* than in ssp. *sinensis* (35.56% vs 31.78%). A higher proportion of palmitic (7.41% vs 6.38%) and oleic (16.37% vs 13.96%) acids and a lower proportion of stearic acid (2.19% vs 2.23%) were discovered between the accessions of ssp. *sinensis* and hybrids. The polyunsaturated fatty acids (PUFA) ratio in hybrids (76.06%) was slightly lower than that in ssp. *mongolica* (77.66%) and higher than that in ssp. *sinensis* (72.22%). Some hybrids (including ZJ1, Za1-2, Za13-25, Za05-6, LFZ, and ZCY12) contained a high proportion of PUFAs (> 80%) in seed oil, which was more than the average level of ssp. *mongolica* accessions (S6 Table).

In pulp oil, the dominant FAs were palmitoleic, palmitic, linoleic, oleic, and vaccenic acids (Table 3). Major differences were observed in the proportion of palmitoleic (17.93-57.75%), oleic (1.44-23.43%) and vaccenic (3.51-24.24%) acids. The special feature of pulp oil is high proportions (> 35%) of palmitoleic and palmitic acids. Compared to ssp. *sinensis*, ssp. *mongolica* contained a higher proportion of palmitoleic and palmitic acids in the berry pulp ($p < 0.05$) (Table 4). In particular, the proportions of oleic and vaccenic acids were highest in ssp. *sinensis*, much higher than those in ssp. *mongolica* and hybrid accessions. The relative levels of α-linolenic and stearic acids in pulp of ssp. *sinensis* were higher than ssp. *mongolica* ($p < 0.05$) (Table 4). For hybrids, the proportions of most FAs were between ssp. *mongolica* and ssp. *sinensis* accessions, except for linoleic acid. Similar to the results in seed oils, the hybrids had the highest proportions of linoleic acid (11.53%) and PUFA (12.60%). The monounsaturated fatty acid (MUFA) ratio in the pulp oil of ssp. *sinensis* (57.00%) was highest and that of ssp. *mongolica* (51.00%) was almost equal to that of the hybrids (51.20%). In the hybrids, the pulp oil of SCY2 contained 39.16% palmitoleic acid, and the content of MUFAs was 60.77%, which was higher than that in ssp. *sinensis* (S6 Table).

Correlations among the agronomic traits and oil characteristics

Canonical analyses allow direct comparisons of two data matrices. All sea buckthorn

accessions were represented in a two-dimensional space using CCA between phenotypic traits and oil characteristics (Fig 3). For berries of the two different subspecies and hybrid accessions, phenotypic characters (BLD, HBW, BSI, and BTD) of berries and oil traits in pulp showed a close correlation (r = 0.8725, p = 0.0000). Based on CCA, accessions of ssp. *mongolica* were clustered on the upper side (mainly positive on D1 and D2), those of ssp. *sinensis* on the other, and the hybrids in the middle in Fig 3A. The positioning of samples in the first dimension was mostly related to differences in their berry characteristics that were primarily provided by a marker of BLD. The second dimension indicated differences in the oil contents and FA compositions of pulp oil among sea buckthorn accessions. Differences between pulp oil traits were primarily related to percentages of oil content, 16:0 and 16:1n7, which were highest in ssp. *mongolica*, followed by hybrids, and lowest in ssp. *sinensis*. For seeds of 78 accessions, phenotypic characteristics (SL, SW, ST, and HSW) and seed oil traits were correlated (r = 0.7482, p = 0.0000). The positioning of samples was staggered (Fig 3B), which reflected that all seed samples had relatively little variation among phenotypic traits and oil characteristics. These results verified the previous analysis (Table 2 and Table 3).

Fig 3. Canonical correspondence analysis of phenotypic traits (A. berry; B. seed) and oil characteristics (A. pulp oil; B. seed oil) of sea buckthorn germplasms. D1, Dimension 1; D2, Dimension 2. ▲ = ssp. *mongolica*; ● = ssp. *sinensis*; ◇ = hybrid.

SSR diversity

Twenty-three pairs of RNA-Seq SSR primers with good amplification and band stability were used ~~in~~ 78 accessions of sea buckthorn. A total of 69 bands were amplified using the 23 primer pairs, of which 59 were polymorphic, accounting for 85.51% of all bands. The number of amplified bands per locus ranged from 2 to 5, averaging 3, and Ne ranged from 1.0392 to 3.1049, averaging 1.6602 (Table 6). SB2, SB3, SB5, SB6, SB8, SB13, SB16 and SB23 were informative SSR loci, each revealing more than four effective alleles distributed among all of the accessions. Compared with Na, Ne and their average values were lower, which was caused by the uneven distribution of gene frequencies in SSR loci. In the genetic diversity analysis, Ho ranged from 0.0385 to 0.7949, with an average of 0.2965; He ranged from 0.0377 to 0.6779, with an average of 0.3291; and Is ranged from 0.0950 to 1.2152, with an average of 0.5681. The PIC value, regarded as discriminating power, varied from 0.0370 to 0.6174, with an average of 0.2845. Loci SB6 (PIC = 0.6174) and SB8 (PIC = 0.5820) showed higher effectiveness because of their high informativity and could be used to construct the fingerprint map of sea buckthorn germplasm. ~~The characteristics of these 23 loci in the genetic diversity analysis of sea buckthorn germplasm are shown in Table 5.~~

Table 5. ~~Characterization of 23 polymorphic SSR markers in the 78 sea buckthorn accessions.~~

Loci code	Na	Ne	Ho	He	PIC	Is
SB1	3	1.2745	0.2436	0.2154	0.2025	0.3956

SB2	4	1.1382	0.1282	0.1214	0.1166	0.2791
SB3	4	2.2372	0.4615	0.5530	0.4627	0.9090
SB4	2	1.5006	0.2692	0.3336	0.2779	0.5160
SB5	4	2.1129	0.3333	0.5267	0.4735	0.9288
SB6	4	3.1049	0.7051	0.6779	0.6174	1.2152
SB7	2	1.0799	0.0769	0.0740	0.0712	0.1630
SB8	5	2.8490	0.3846	0.6490	0.5820	1.1890
SB9	2	1.1509	0.1410	0.1311	0.1225	0.2550
SB10	3	1.5350	0.2949	0.3485	0.3114	0.6253
SB11	2	1.9287	0.1667	0.4815	0.3656	0.6745
SB12	3	1.2430	0.2179	0.1955	0.1753	0.3687
SB13	4	2.1644	0.4231	0.5380	0.4392	0.8687
SB14	2	1.9987	0.3077	0.4997	0.3750	0.6928
SB15	2	1.0662	0.0641	0.0620	0.0601	0.1418
SB16	4	1.4567	0.1923	0.3135	0.2956	0.6427
SB17	2	1.4175	0.3590	0.2945	0.2512	0.4706
SB18	2	1.0392	0.0385	0.0377	0.0370	0.0950
SB19	3	1.0804	0.0641	0.0744	0.0724	0.1804
SB20	2	1.1803	0.1667	0.1528	0.1411	0.2868
SB21	3	1.9123	0.7308	0.4771	0.3802	0.7318
SB22	3	1.2905	0.2564	0.2251	0.2025	0.4084
SB23	4	2.4239	0.7949	0.5874	0.5102	1.0284

380 Na, observed number of alleles; Ne, effective number of alleles; Ho, observed heterozygosity; He,

381 expected heterozygosity; PIC, polymorphism information content; Is, Shannon’s information index.

Genetic relationships among sea buckthorn germplasm

Using 23 polymorphic SSR markers, the UPGMA dendrogram based on the proportion of shared alleles was constructed to assess the genetic relationships between the 78 accessions (Fig 4). The results showed that all the accessions could be divided into two groups (I and II). The accessions of ssp. *sinensis* (JD, ZGSJ, MHC, ZGSJ^{wild}, JPDH and ZXY) were clustered into group I. These accessions had closer relationships, despite great geographic differences. The second group was divided into 3 subgroups, namely, IIa, IIb, and IIc. The 20 hybrid accessions were all clustered into IIa. Subgroups IIb and IIc contained all the accessions of ssp. *mongolica* (introduced from Russia and Mongolia). Subgroup IIb included 6 accessions, namely WCF, LS1, QYSK, FX, SR, and MZ14. The remaining accessions of ssp. *mongolica* were clustered into IIc. Among them, KTN, WLGM, HS4, HS9, HS10, HS12, HS14, HS18, HS20, HS22, WCF, FX and MZ14 composed one sub-subgroup. SJ3, ZYH, SD, NR, FL, XE2, XE3, JYH and YLSD12 showed close relationships. The other 23 accessions clustered into the third sub-subgroup. Overall, the relationship between ssp. *mongolica* and ssp. *sinensis* was relatively distant. The hybrids are close to ssp. *mongolica*, to which their female parents belonged.

Fig. 4. UPGMA dendrogram of sea buckthorn germplasm based on SSR data (sample abbreviations described in Table 1). ▲ = ssp. *mongolica*; ● = ssp. *sinensis*; ◇ = hybrid.

Discussion

Morphological characteristics, biochemical traits, and microsatellite markers have been used for germplasm identification and genetic diversity analysis in many horticultural plants [26−27]. The diversity at the morphological, biochemical, and molecular levels of 78 sea buckthorn accessions, composed of 52 from ssp. *mongolica*, 6 from ssp. *sinensis*, and 20 hybrids, was investigated.

The morphological characterization of plant materials with desired traits is an essential step for the effective use of germplasm [28]. Here, 8 important agronomic traits were measured among 78 sea buckthorn accessions, and a considerable amount of variation in morphological traits was found. The sizes of berries from the two different subspecies and hybrid accessions were significantly different according to the HBW value (p = 0.000). Compared to ssp. *sinensis* berries, ssp. *mongolica* berries were much larger on average. The berry size of hybrid accessions was between the two subspecies. In the PCA, we plotted 2D plots with PC1 and PC2 scores of phenotypes (Fig 2). PC1 was mainly related to BTD and HBW, which explained the largest portion of the variance in 78 accessions. The distribution of 78 accessions on PC1 and PC2 was consistent with their agronomic characteristics (Fig 2). These results estimating morphological traits are valuable tools for identifying variation among plant germplasms [26].

For biochemical traits, oil content and FA composition in the seeds and seedless parts were selected for their important roles in human health. The oil of sea buckthorn seems to be a good source of unsaturated FAs. Seed oil is rich in PUFAs, including

426 linoleic and α-linolenic acids. The proportion of PUFAs did not differ significantly
427 among berries from three origins, despite the differences in some morphological
428 characteristics and in growth conditions. These results were consistent with previous
429 studies [10]. The results of the present study and previous investigations also suggested
430 that the berries of ssp. *mongolica* were a good source of palmitic and palmitoleic acids
431 in pulp oil and that those of ssp. *sinensis* were a good source of oleic acid in both seeds
432 and fruit pulp [29]. Although carefully selected for intersubspecies crosses, some
433 hybrids displayed elite oil traits. For example, the proportion of MUFAs in the pulp of
434 SCY2 and of PUFAs in the seeds of 6 accessions (including ZJ1, Za1-2, Za13-25, Za05-
435 6, LFZ, and ZCY12) exceeded the average level of ssp. *mongolica* accessions, the
436 subspecies that one of their parents belonged to. These results demonstrate the
437 effectiveness of traditional cross breeding in the improvement of native accessions (ssp.
438 *sinensis*), even though it is time-consuming and has low efficiency.

439 Previous studies found that berry size is a useful indicator of Vc, sugars and acids
440 in population identification [19, 30]. The nutrients in the seedless fraction were more
441 concentrated in the small berries of ssp. *sinensis* than in the large berries of ssp.
442 *mongolica* [29]. In the present study, we analyzed the correlation between agronomic
443 characteristics and oil traits at different levels (seed and pulp) by CCA. The results
444 showed that the phenotypic characteristics (BLD, HBW, BSI, and BTD) of berries and
445 the oil traits in pulp were positively correlated (r = 0.8725, p = 0.0000). The BLD, as a
446 promising marker, provided the primary difference in CCA. Our results illustrated that
447 berry size had different correlations with various biochemical characteristics in sea

448 buckthorn.

449 Variation in phenotypic traits among germplasms may be attributed to differences
450 in genetic backgrounds, geographical location, climate, harvest period and berry
451 maturity, while molecular markers are independent of environmental condition and
452 growth stage [31]. Twenty-three polymorphic SSR markers were used to identify 78 sea
453 buckthorn accessions. The 23 selected SSR markers detected 2–5 alleles, and their PIC
454 values ranged from 0.1166 to 0.6155 and had an average of 0.3249. The PIC mean value
455 was significantly lower than that of RAPD, ISSR and SRAP markers previously
456 reported [15−16, 32], suggesting that the gene sequences of these SSR markers were
457 conserved in sea buckthorn germplasm.

458 Based on UPGMA, the 78 accessions were classified into two groups. There is a
459 large genetic distance between accessions of ssp. *sinensis* and ssp. *mongolica*. The
460 hybrids were in between and rather close to ssp. *mongolica* accessions. Coincidentally,
461 these hybrids were also between ssp. *sinensis* and ssp. *mongolica* accessions on the
462 PCA plot based on 8 agronomic characteristics. This result illustrated that the diversity
463 of morphological characteristics could reflect genetic diversity and be used as markers
464 in agronomy. Ruan et al. [15] assessed 14 Chinese, Russian and Mongolian sea
465 buckthorn accessions using RAPD markers and obtained similar results. In a previous
466 publication, the genetic relationship of 31 sea buckthorn accessions (also contained in
467 this study) was analyzed based on 17 RNA-Seq SSRs [14]. However, the accessions of
468 ssp. *mongolica* clustered in one group and those of ssp. *sinensis* and hybrids were
469 divided in the other. This revealed that genetic relationships mainly relied on the

diversity of genotypes and genetic backgrounds.

With the continuous development of high-throughput sequencing technology, transcriptome databases have become a powerful resource for SSR mining. An increasing number of RNA-Seq SSRs have been developed and applied to the study of species genetic diversity and population genetic structure [33−34]. The SSRs obtained by transcriptomes are associated with many important quantitative traits [35].

The results in the present study yielded useful knowledge regarding the diversity and genetic relationships of sea buckthorn germplasm in northern China, and could therefore facilitate further studies, including the selection of mapping populations and promising candidates, marker-trait association analysis based on establishing the consistency of the traits , and characterization of parents used in future breeding programs.

Conclusion

In the present study, 8 phenotypic characteristics, oil traits in seeds and seedless parts, and 23 SSR markers successfully distinguished all 78 sea buckthorn accessions. In PCA, BTD and HBW in the first PC were the most important characteristics for distinguishing the accessions. The agronomic traits of berries were closely correlated with the oil content and FA composition in the pulp by CCA. This information will be valuable for germplasm identification and genotypic diversity analysis in *~~Hippophae~~ rhamnoides*.

Supporting information

S1 Fig. 78 berry samples used in this study. Numbers are the variety codes listed in Table 1.

(TIF)

S2 Fig. Total ion flow chromatography of 37 FAMEs Mix (A) and FAMEs in pulp oil in MHC (B).

(TIF)

S1 Table. Samples of sea buckthorn grouped according to different genetic backgrounds.

(DOCX)

S2 Table. Characterization of the hybrids of sea buckthorn accessions studied.

(DOCX)

S3 Table. Climatic conditions at different growth sites of sea buckthorn samples in China.

(DOCX)

S4 Table. Primer sequences, annealing temperature, and estimated allelic size of 23 SSR markers.

(DOCX)

S5 Table. Descriptive statistics for morphological traits of berries and seeds among the sea buckthorn accessions studied.

(DOCX)

S6 Table. The morphological characteristics and oil traits of pulp and seeds of the 78 sea buckthorn accessions studied.

(XLSX)

S7 Table. Fruit traits and Vc contents of large berry accessions of sea buckthorn in two experimental fields (located in Suiling and Dengkou).

(DOCX)

S8 Table. Allele combinations obtained at the 20 microsatellite loci in 78 sea buckthorn accessions.

(TXT)

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Figure

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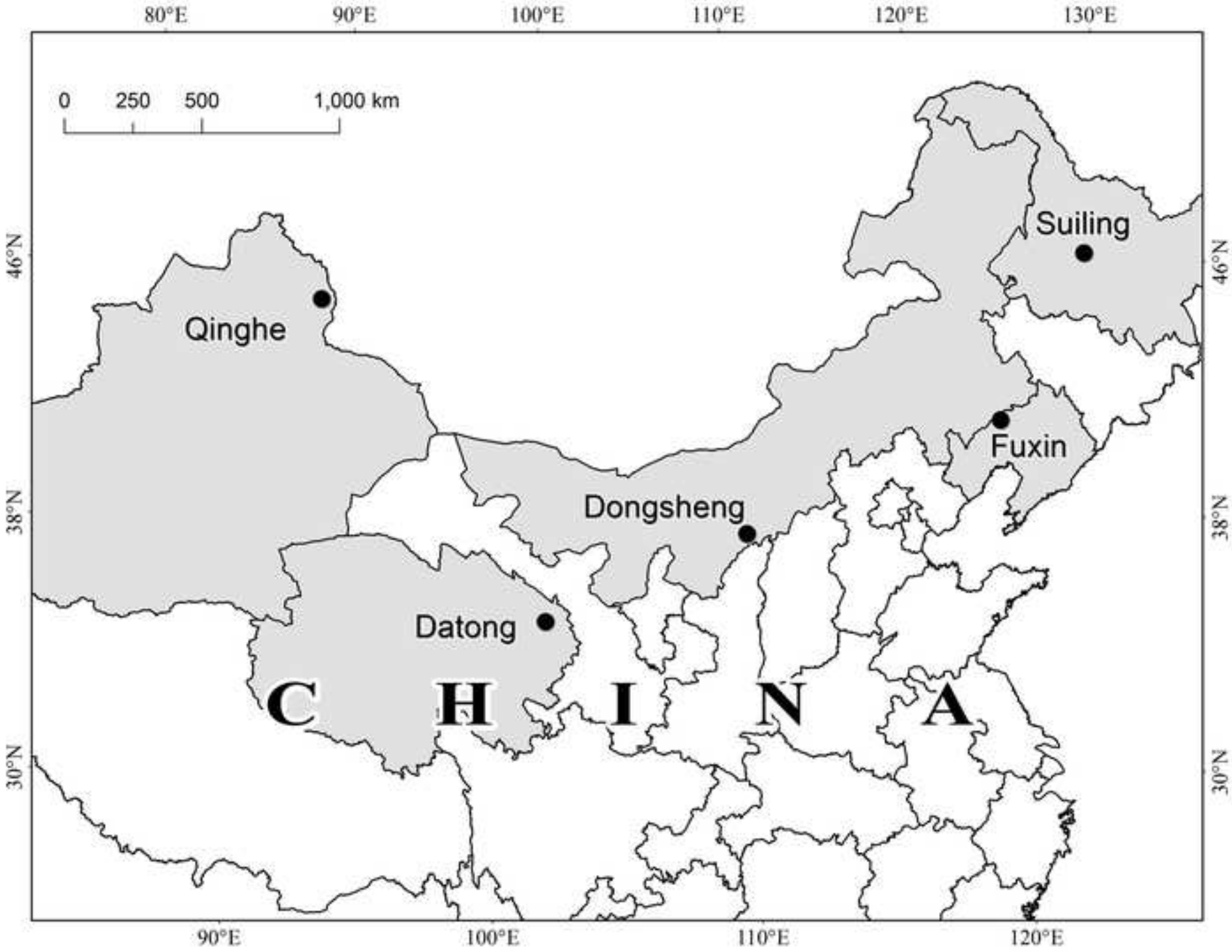

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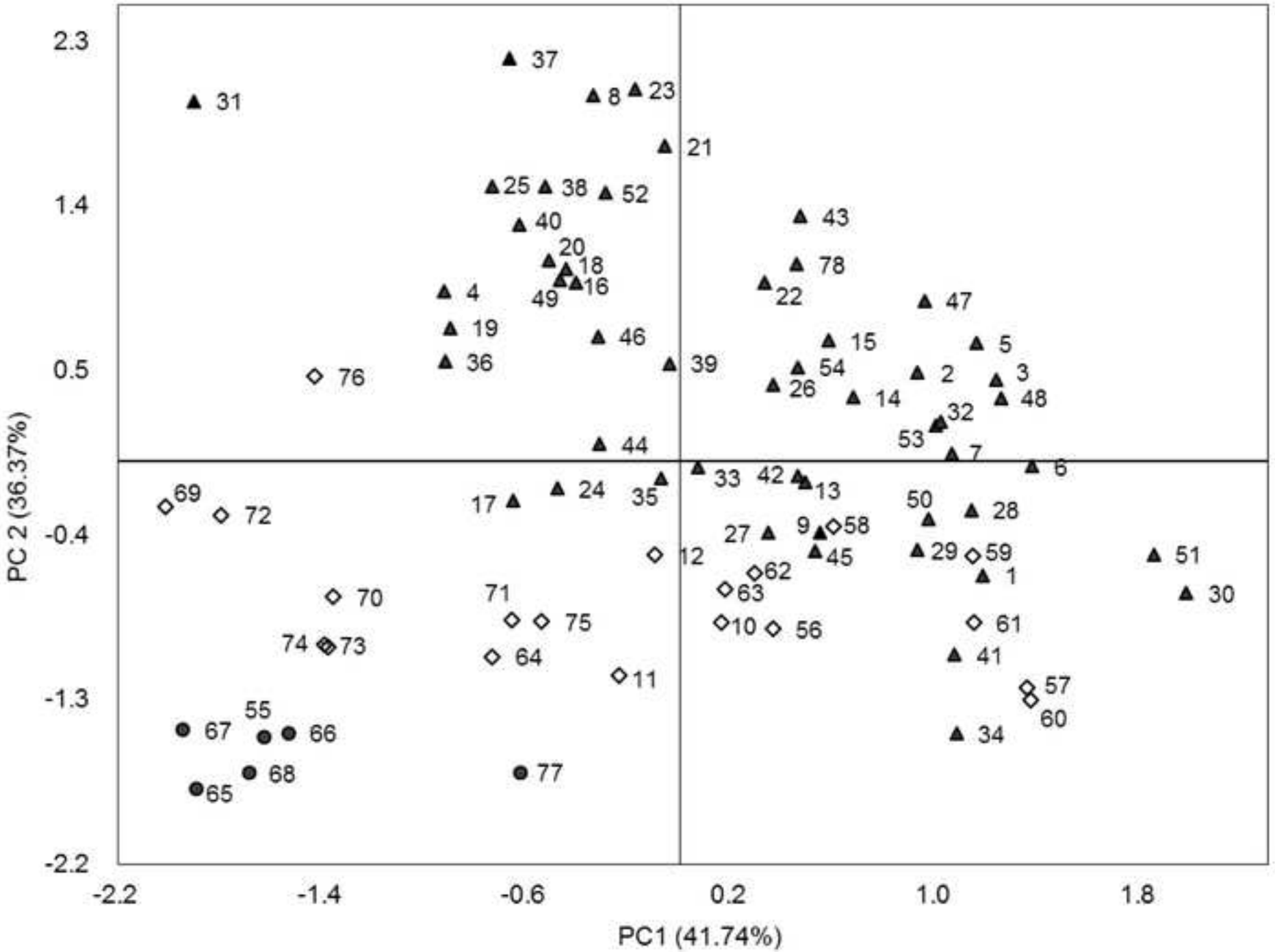

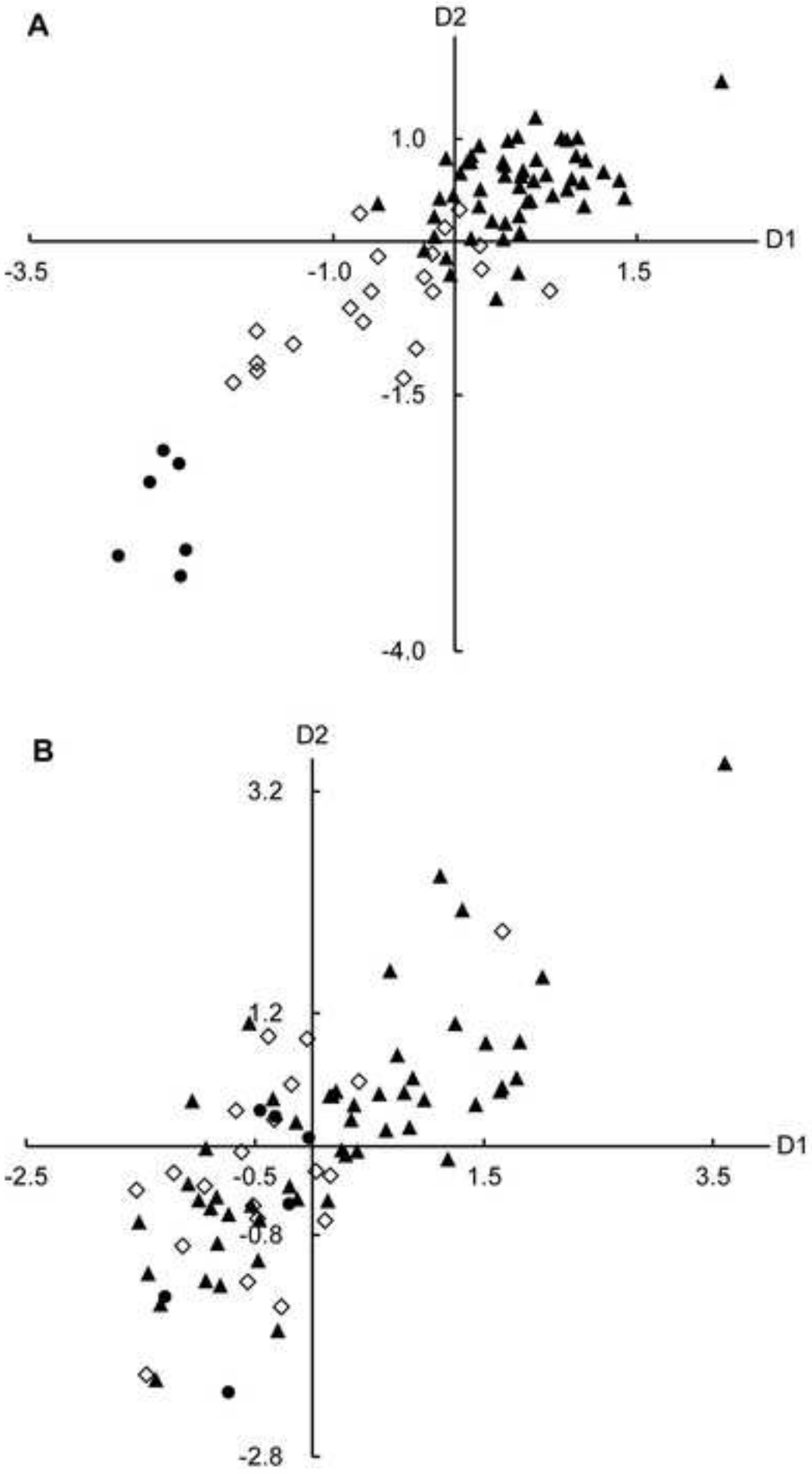

Figure

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