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Assessment of Genetic Diversity and Protein Content of Scandinavian Peas (*Pisum sativum*)

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Abstract: We produced homogeneous lines of 227 pea accessions from the Nordic Genetic Resource Center via single seed descent. The genetic diversity among these, mostly Scandinavian accessions, was investigated using three microsatellite markers, A9, AC58 and AA5. The microsatellites were highly informative and separated 153 of 194 accessions on a Neighbor Joining topology. The high polymorphism information content (*PIC*) values between 0.87 and 0.91 indicated that the gene bank material contains a large number of pea accessions with different breeding histories. The peas were grown in the field for two years and seed protein content showed variation between 9.3% and 34.1% over the years and accessions, respectively. The mean thousand seed weight was 152.05 g. More than 10 accessions had a protein content above 28%, showing that the collection has potential as breeding nursery for high-protein pea cultivars.

Keywords: pea; *Pisum sativum* L.; SSR markers; *PIC*; microsatellites; genetic diversity; Neighbor Joining tree; protein content; thousand seed weight

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1. Introduction

Peas (*Pisum sativum* L.) have been grown in Denmark since 500–1500 AD and sweet and soft pea types for human consumption since 1600 [1]. *Pisum sativum*, originating in the Mediterranean region, has been adapted to the temperate climate and long days of Denmark. The world production of peas is 10.5 mill hectares [2]. *Pisum sativum* belongs to the legume family, Fabaceae, and is a self-pollinated, diploid species with 2n = 14 and a very large genome of 3.92 Gb [3]. Peas were the model plant used by Gregor Mendel to discover general rules for genetic heredity [4]. Peas are currently an important crop for food, feed and sidechain products, with a great potential for future farming. The content of dietary proteins in different pea cultivars ranges from 24.3 to 32.6%. The starch content is between 33.4 and 47.5% and peas contain fibers as well as mineral nutrients [5,6]. Others found that the protein content of pea cultivars varies with environmental and agronomic factors [7–9] with a typical span in protein content from 14.5% to 28% [10,11]. The symbiosis with nitrogen-fixing soil bacteria favors the growing of peas in low-input farming systems because it reduces the need for nitrogen fertilizers, and when used in crop rotation, peas add nitrogen to the soil [3,12].

The genetic diversity in peas has been studied using microsatellites also named simple sequence repeats (SSR). A worldwide collection of 372 pea cultivars and landraces has been analyzed using 29 different microsatellite markers [13]; a collection of 175 pea cultivars from the Czech Republic has been analyzed using seven microsatellite markers [14], and 130 pea landraces from Turkey were analyzed using 14 markers [15]. The genetic diversity measured as polymorphism information content (*PIC*) ranges from 0.15 to 0.96 for different types and numbers of microsatellites [15]. The microsatellite technique is also efficient when analyzing smaller dedicated pea collections such as garden peas, forage peas, landraces and historical collections from various countries, e.g., Tunisia, Anatolia, Australia, India, and from gene banks [16–25]. These studies included between 8 and 34 microsatellites showing

PIC values ranging from 0.19 to 0.84. Specific microsatellite markers are developed for a collection of field pea cultivars from Ethiopia, which also identify private alleles [26]. A recent study of a forage pea collection of landraces from Anatolia successfully differentiated the accessions into subgroups of locally adapted genotypes [17]. With the SSR markers already available in the literature cited above, the initial cost of employing SSR markers to the present study is low.

The polymorphic information content (*PIC*) describes the polymorphism and thus the informational value of the microsatellite. Generally, *PIC* values above 0.5 are highly informative, between 0.25 and 0.5 reasonably informative, and below 0.25 slightly informative [27]. In a study based on the pea genome, 309 microsatellites were constructed and 235 were polymorphic in either the first or second set of tests [28]. The number of PCR fragments (alleles) per microsatellite is between two and seven. The *PIC* value of the microsatellites varies from 0.04 to 1 with a mean of 0.63, thus showing a wide range of information from slightly to highly informative [27,28]. A comparison of the genetic variation and morphological features of 65 pea cultivars and 21 wild pea accessions [29] found 51% polymorphic information content and the dendrogram shows that diversity among the varieties from Europe is narrower than in the rest of the accessions. Furthermore, the study supports the assumption that the topology of the dendrogram reflects the pattern of refinement of the cultivars [29]. In an analysis of 19 pea cultivars primarily from Australia and Russia, five of eight microsatellites successfully produced 34 PCR fragments in total with 3 to 13 fragments per microsatellite [30]. The *PIC* values ranged from 0.18 to 0.79 with a mean of 0.62 and classified the microsatellites as highly informative. The genetic diversity among 35 pea accession was assessed with 15 microsatellites, which produced 41 different fragments, and all genotypes were identified on a UPGMA tree [31]. The *PIC* values ranged from 0.05 to 0.66 with a mean of 0.46. Jain et al. [32] investigated the genetic diversity among 96 cultivars from all over the world using 31 microsatellites along with 42 expressed sequence tag markers and 11 modern markers. The microsatellites identified 83 PCR fragments with an average of 2–6 per microsatellite and the *PIC* values varied from 0.01 to 0.56 with a mean of 0.29. Nasiri et al. [33] investigated 77 pea accessions from 17 countries using 10 microsatellites. The results included 59 PCR fragments, which varied from 2 to 8 per microsatellite. The *PIC* values ranged between 0.56 and 0.84, with a mean of 0.72. Kumari et al. [34] investigated 28 pea genotypes with 32 microsatellites, which resulted in 44 fragments with 2 to 4 fragments per microsatellites. The *PIC* values were between 0.31 and 0.66 with a mean of 0.49.

The current study surveyed a subset of the approximately 2500 pea accessions of Peas at the Nordic Genetic Resource Center, NordGen, https://www.nordgen.org/ (accessed on 16 March 2023), many of which were from the Weibullsholm *Pisum* collection. The accessions were primarily chosen based on prior knowledge of potential high protein content. The investigations were a first step in a pre-breeding process towards selecting and developing pea cultivars with a high protein content to meet the demand for plant-based protein for human consumption and as an alternative source of protein for husbandry. We address the following three questions: (1) What is the genetic diversity among the accessions and is the diversity structured? (2) What is the polymorphic information content? (3) What is the level of protein content and the thousand seed weight of accessions?

2. Materials and Methods

2.1. Plant Material

The plant material included in this study originated from the Nordic Genetic Resource Center, https://www.nordgen.org/ (accessed on 16 March 2023; Table 1. Most accessions originated from the Nordic countries and information on the specific location of origin, pedigree relationships, and proper cultivar name is in general sparse but some information may be retrieved in the seed database at https://www.nordic-baltic-genebanks.org/gringlobal/search.aspx (accessed on 16 March 2023). A total of 227 pea accessions were

grown from a single seed in pots in the greenhouse. Plants were grown to maturity and the seeds were collected for further studies in the field.

Table 1. List of *Pisum sativum* accessions included in this study. SKR is the working number given to the accession in this study. Accession is the number from NordGen, www.NordGen.org (accessed on 16 March 2023). Name included cultivar name, if existing, WBH designate a pre-breeding line from Weibuhlholm. AA5, AC58 and AA9 were the microsatellites used and x indicate that the accession produced a result. TSW were the thousand seed weight and then seed protein content in % for 2017, 2018 and 2019.

SKR. No.	Accession	Name	AA5	AC58	AA9	TSW 2017 (g)	Protein 2017 (%)	Protein 2018 (%)	Protein 2019 (%)
SKR1	NGB103441	WBH 3441	x	x	x	178.1	23.4	26.9	
SKR2	NGB101418	WBH 1418	x	x	x	107.3	20.3	20.8	
SKR3	NGB103563	WBH 3563	x	x	x		16.0		
SKR4	NGB101515	WBH 1515	x	x	x				
SKR5	NGB100756	WBH 756	x	x	x	185.0	25.0	29.9	25.0
SKR6	NGB101687	WBH 1687	x	x	x		19.7		
SKR7	NGB105795	multimicrodentatus	x	x	x		15.6		
SKR8	NGB103423	Latah		x	x		14.3		
SKR9	NGB100463	WBH 463	x	x	x		16.0		
SKR10	NGB103565	WBH 3565	x	x	x		12.0		
SKR11	NGB101735	Improved Harbinger	x	x	x	244	21.0	25.6	
SKR12	NGB101736	rouge C15	x	x	x		17.7		
SKR13	NGB101339	Bonneville	x	x	x	180.2	26.4	22.0	
SKR14	NGB101741	New Season	x	x	x		19.5		
SKR15	NGB101603	WBH 1603	x	x	x				
SKR16	NGB103546	W.S.U.-28	x	x	x		22.3	25.9	
SKR17	NGB101772	Wellensiek's tester	x	x	x		18.6		
SKR18	NGB101325	WBH 1325	x	x	x				
SKR19	NGB101330	WBH 1330	x	x	x		20.0		
SKR20	NGB101165	WBH 1165	x	x	x	142.2	23.0	24.1	
SKR21	NGB103436	WBH 3436	x	x	x		18.3		
SKR22	NGB102963	Wilt Resistant Thomas Laxton				172.8	24.7	25.8	
SKR23	NGB101889		x	x	x				
SKR24	NGB103439	WBH 3439	x	x	x		13.9		
SKR25	NGB103429	WBH 3429	x	x	x		20.6	24.1	
SKR26	NGB103583	WBH 3583	x	x	x				
SKR27	NGB100592	WBH 592		x	x				
SKR28	NGB103452	WBH 3452	x	x	x				
SKR29	NGB102158	WBH 2158	x	x	x	135.5	18.6	21.5	
SKR30	NGB103422	Alaska	x	x	x		18.2		

Table 1. *Cont.*

SKR. No.	Accession	Name	AA5	AC58	AA9	TSW 2017 (g)	Protein 2017 (%)	Protein 2018 (%)	Protein 2019 (%)
SKR31	NGB103449	Feltham First	x	x	x	178.9	21.5	24.4	
SKR32	NGB103426	Juneau	x	x	x		17.8		
SKR33	NGB103442	WBH 3442	x	x	x		14.4		
SKR34	NGB103576	WBH 3576	x	x	x	187	31.7		
SKR35	NGB103578	WBH 3578	x	x	x	193	25.6	26.1	20.5
SKR36	NGB100851	procumbens	x	x	x	165	23.4	28.5	
SKR37	NGB101338	Salzmunder Edelperle	x	x	x		17.1		
SKR38	NGB103433	WBH 3433	x	x	x		16.6		
SKR39	NGB102177	WBH 2177	x	x	x	132	23.7	27.3	
SKR40	NGB103438	WBH 3438	x	x	x		20.1		
SKR41	NGB101608	WBH 1608	x	x	x				
SKR42	NGB103435	WBH 3435	x		x		12.1		
SKR43	NGB102058	WBH 2058	x	x	x		20.0		
SKR44	NGB101524	patelliformis	x	x	x				
SKR45	NGB101570	WBH 1570	x	x	x		13.1		
SKR46	NGB103568	WBH 3568	x	x	x	64.4	21.7	22.2	
SKR47	NGB103581	WBH 3581	x	x	x				
SKR48	NGB105136	chlorotica	x	x	x	76.4	30.0	22.9	24.4
SKR49	NGB102184	New Era	x	x	x		16.6		
SKR50	NGB102160	WBH 2160	x	x	x	144.4	22.4	28.5	
SKR51	NGB103580	WBH 3580	x	x	x	156	23.7	21.3	
SKR52	NGB102022	WBH 2022	x	x	x	104	30.2	23.8	
SKR53	NGB102663	WBH 2663	x	x	x				
SKR54	NGB102203	WBH 2203	x	x	x		15.6		
SKR55	NGB102217	chlorotica	x	x	x		19.9		
SKR56	NGB105350	/chlorina				157.2	27.3	24.8	
SKR57	NGB102431	Laxtonian				193	23.8	24.8	24.1
SKR58	NGB105124	ageotropum	x	x	x		17.0		
SKR59	NGB102496		x	x	x	151	32.1		
SKR60	NGB102214	chlorotica	x	x	x	123	23.3	28.9	
SKR61	NGB102210	chlorotica	x	x	x	145	23.8	24.3	
SKR62	NGB102136	WBH 2136	x	x	x	162	25.4	26.3	
SKR63	NGB102216	chlorotica	x	x	x	104.8	29.4	30.6	24.3
SKR64	NGB105862	densinodosum				155	20.9	34.1	
SKR65	NGB105428	chlorotica	x	x	x		15.6		
SKR66	NGB102574	Beta	x	x	x	150.1	22.4	24.6	
SKR67	NGB102622		x	x	x	164	22.4	24.9	21.1
SKR68	NGB102988	WBH 2988	x	x	x		20.2		
SKR69	NGB106051	reduced in wax	x	x	x	166.6	21.4	29.0	

Table 1. *Cont.*

SKR. No.	Accession	Name	AA5	AC58	AA9	TSW 2017 (g)	Protein 2017 (%)	Protein 2018 (%)	Protein 2019 (%)
SKR70	NGB102432	Hundredfold	x	x	x		18.2		
SKR71	NGB105789	/compactum	x	x	x		19.0		
SKR72	NGB106060	supaeromaculata					16.3		
SKR73	NGB102239		x	x	x		20.3		
SKR74	NGB102369	WBH 2369	x	x	x	129.2	21.9	25.2	
SKR75	NGB105051	/compactum	x	x	x				
SKR76	NGB105432	reductus	x	x	x	84	26.2	25.1	
SKR77	NGB106080	WBH 6080	x	x	x		17.4		
SKR78	NGB102579		x		x	231	27.0	23.1	
SKR79	NGB102581		x	x	x		24.5		
SKR80	NGB105765	chlorotica				132	20.9	26.1	
SKR81	NGB103431	WBH 3431	x	x	x		18.1		
SKR82	NGB102823	Austrian Winter	x	x	x		13.1		
SKR83	NGB103571	WBH 3571	x	x	x		14.4		
SKR84	NGB103572	WBH 3572	x	x	x				
SKR85	NGB103573	WBH 3573	x	x	x		17.3		
SKR86	NGB103585	WBH 3585	x	x	x		13.5		
SKR87	NGB101452	WBH 1452	x	x	x	159	22.3	22.0	
SKR88	NGB101192	WBH 1192				152	28.3	24.6	24.8
SKR89	NGB101391	WBH 1391	x	x	x				
SKR90	NGB101689	WBH 1689	x	x	x		25.1		
SKR91	NGB101017	WBH 1017	x	x	x	151.4	24.9	28.9	
SKR92	NGB101351	WBH 1351	x	x	x				
SKR93	NGB105534	supaeromaculata 68	x	x	x	113.1	28.9	28.7	23.4
SKR94	NGB102188	WBH 2188	x	x	x	181.87	22.6	27.7	
SKR95	NGB103420	WA 788	x	x	x		14.6		
SKR96	NGB103434	WBH 3434					18.3		
SKR97	NGB103421	Lilaska		x	x		18.9		
SKR98	NGB103428	WBH 3428					18.5		
SKR99	NGB103432	WBH 3432		x	x		19.6		
SKR100	NGB102070	Puke		x	x		16.2		
SKR101	NGB103577	WBH 3577	x	x	x	174.8	21.9	26.0	
SKR102	NGB100909	WBH 909				160	22.0	24.0	
SKR103	NGB101500	WBH 1500		x	x	99.1	20.5	26.3	
SKR104	NGB103437	WBH 3437				67	23.7	24.6	
SKR105	NGB102069	Patea	x	x	x	212.4	22.0	19.4	
SKR106	NGB103561	WBH 3561		x	x		21.2		
SKR107	NGB103567	WBH 3567	x	x	x		17.0		
SKR108	NGB105310	cerosa	x	x	x	108.8	26.4	27.0	22.5

Table 1. *Cont.*

SKR. No.	Accession	Name	AA5	AC58	AA9	TSW 2017 (g)	Protein 2017 (%)	Protein 2018 (%)	Protein 2019 (%)
SKR109	NGB103574	WBH 3574	x	x	x				
SKR110	NGB103575	WBH 3575	x	x					
SKR111	NGB103579	WBH 3579	x	x	x		17.0		
SKR112	NGB103584	WBH 3584	x	x	x				
SKR113	NGB103602	WBH 3602	x	x	x		11.2		
SKR114	NGB103603	WBH 3603	x	x	x		18.7		
SKR115	NGB102901		x	x	x		12.8		
SKR116	NGB103430	WBH 3430	x	x	x		18.3		
SKR117	NGB103427	WBH 3427	x	x			17.1		
SKR118	NGB102159	WBH 2159	x	x			18.5		
SKR119	NGB102844		x		x		19.2		
SKR120	NGB102185	New Wales	x	x	x	209	24.6	25.4	23.0
SKR121	NGB101132	WBH 1132	x	x	x				
SKR122	NGB103570	WBH 3570	x	x	x		19.5		
SKR123	NGB103582	WBH 3582		x	x		18.7		
SKR124	NGB103604	WBH 3604	x	x	x		12.4		
SKR125	NGB103605	WBH 3605	x	x			13.3		
SKR126	NGB101784	Mrkos horizontale	x	x	x				
SKR127	NGB102063	WBH 2063	x	x	x	172	24.4	26.9	20.7
SKR128	NGB105765	chlorotica				125.1	26.1	25.1	
SKR129	NGB102578		x		x	148	33.6	26.0	21.2
SKR130	NGB102537						19.1		
SKR131	NGB103458		x		x	118.4	20.6	26.7	22.2
SKR132	NGB105161	costata	x	x	x	92	25.8	23.3	
SKR133	NGB102999	WBH 2999	x	x	x		17.4		
SKR134	NGB102687	WBH 2687	x	x		178.5	21.2	26.9	22.7
SKR135	NGB105267	variomaculata	x		x	148	27.3	29.8	22.0
SKR136	NGB105814	chlorotica	x	x	x	118	21.9	26.8	
SKR137	NGB102037	WBH 2037	x	x	x		17.2		
SKR138	NGB102190	WBH 2190	x		x	223	28.8	26.1	22.4
SKR139	NGB102763	Wonder Van Amerika I2048	x	x	x	142	22.2	27.4	
SKR140	NGB106000	vixcerata				232	29.4	28.6	23.0
SKR141	NGB102582						19.5		
SKR142	NGB105449	variomaculata	x	x	x	120.2	22.9	27.5	
SKR144	NGB102370	WBH 2370	x	x	x		22.2		
SKR145	NGB102621		x	x	x		19.2		
SKR146	NGB102927		x	x	x		17.5		
SKR147	NGB103459		x	x	x	150	25.5	25.7	

Table 1. *Cont.*

SKR. No.	Accession	Name	AA5	AC58	AA9	TSW 2017 (g)	Protein 2017 (%)	Protein 2018 (%)	Protein 2019 (%)
SKR148	NGB105820	/xantha	x	x	x				
SKR149	NGB102521		x	x	x	238	20.5	27.3	22.4
SKR150	NGB102617						16.8		
SKR151	NGB103457		x	x	x	144	22.8	21.7	
SKR152	NGB105410	variomaculata	x	x	x	130.6	26.2	26.9	23.3
SKR153	NGB105981	precocious yellowing	x	x	x	107	24.5	29.1	23.6
SKR154	NGB102480		x	x	x	124.4	25.5	29.5	21.4
SKR155	NGB102588		x	x	x				
SKR156	NGB105961	chlorotica	x	x	x	156.6	23.2	29.0	
SKR157	NGB102205	chlorotica	x	x	x	134	22.2	25.8	
SKR158	NGB102497					193	31.6		
SKR159	NGB102533						19.7		
SKR160	NGB102831		x	x	x	149	24.2	25.2	
SKR161	NGB103052	WBH 3052	x	x	x	123	28.7	27.5	24.0
SKR162	NGB103061	WBH 3061	x	x	x				
SKR163	NGB103116		x	x	x		19.4		
SKR164	NGB105261	variomaculata	x	x	x	150	24.3	33.1	20.0
SKR165	NGB105806	chlorotica	x	x	x	131	23.5	25.7	22.5
SKR166	NGB105983	precocious yellowing				90	25.6	26.9	23.2
SKR167	NGB102005	Nischkes Riesengefärgs Wi. Erbse	x	x	x		17.2		
SKR168	NGB100993	WBH 993	x	x	x	173	27.9	32.0	19.9
SKR169	NGB103559	WBH 3559	x	x	x		12.7		
SKR170	NGB102405	Supergrade	x	x	x	194	21.9	24.6	21.8
SKR171	NGB101742	New Wales	x	x	x	182	22.5	29.9	
SKR172	NGB101888		x	x	x				
SKR173	NGB102423	Senator	x	x	x		18.4		
SKR174	NGB103560	WBH 3560					10.9		
SKR175	NGB102429	Gradus					19.7		
SKR176	NGB103569	WBH 3569	x	x	x		12.9		
SKR177	NGB103545	Puget	x	x	x				
SKR178	NGB103566	WBH 3566	x	x	x		16.2		
SKR179	NGB103484	Dhamar	x	x	x				
SKR180	NGB103425	WBH 3425	x	x	x		18.7		
SKR181	NGB103547	Grant	x	x	x	145	22.4	25.6	
SKR182	NGB102130	WBH 2130	x	x			9.3		
SKR183	NGB103609	WBH 3609					17.2		
SKR184	NGB101979	Ambrosia	x	x	x	144	24.0	27.4	21.8

Table 1. *Cont.*

SKR. No.	Accession	Name	AA5	AC58	AA9	TSW 2017 (g)	Protein 2017 (%)	Protein 2018 (%)	Protein 2019 (%)
SKR185	NGB100800	Primus	x	x	x	149	22.5	26.8	
SKR186	NGB101721	Midfreezer	x	x	x		19.1		
SKR187	NGB101395	WBH 1395	x	x	x	148	24.4	27.1	23.1
SKR188	NGB101677	Mexique 4	x	x	x		16.7		
SKR189	NGB103440	WBH 3440	x	x	x	143	22.7	26.8	
SKR190	NGB101462	WBH 1462	x	x	x		25.0	24.2	
SKR191	NGB101362	clavicula	x	x	x	156	17.0		
SKR192	NGB100640	WBH 640	x	x	x		18.4		
SKR193	NGB103450	Meteor	x	x	x		18.6		
SKR195	NGB103562	WBH 3562	x	x	x	164	20.9	26.5	
SKR196	NGB101463	Sigyn	x	x	x	132	23.2	24.9	
SKR198	NGB101341	Klema Vereduna	x	x	x		20.1		
SKR199	NGB103424	WBH 3424	x	x	x		16.2		
SKR200	NGB103544	Ranger	x	x		214	21.7	25.2	
SKR201	NGB103564	WBH 3564	x	x	x		16.9		
SKR202	NGB103610	WBH 3610	x		x		15.7		
SKR203	NGB105454	viridis	x	x	x	161	22.8	25.3	
SKR204	NGB105995	vixcerata	x	x	x	134	29.0	28.8	26.4
SKR205	NGB102212	chlorotica	x	x	x		18.7		
SKR206	NGB102688	WBH 2688	x	x	x				
SKR207	NGB105271	costata	x	x	x		17.2		
SKR208	NGB105565	chlorotica	x	x	x		23.7		
SKR209	NGB105848	fasciata	x	x	x		26.0		
SKR210	NGB106116	narrow leaflet base	x	x	x		21.1		
SKR211	NGB102178	Thomas Laxton	x	x	x	225	22.0	33.0	
SKR212	NGB102737	WW 709	x	x	x		17.0		
SKR213	NGB102071	Piri	x	x	x		18.6		
SKR214	NGB102183	Darkskin Perfection	x	x	x	154	22.2	25.2	
SKR215	NGB103451	Lilaska	x	x	x		16.7		
SKR216	NGB102072	Pania	x	x		175	20.8	27.4	
SKR217	NGB103606	WBH 3606	x	x	x				
SKR218	NGB103607	WBH 3607		x	x	254.2	23.9	26.3	
SKR219	NGB103628	ramosus	x	x	x	96	21.6	25.1	
SKR220	NGB101304	WBH 1304-1				159	22.4	27.9	
SKR221	NGB101304	WBH 1304-2					16.1		
SKR222	NGB101304	WBH 1304-3				150	22.4	27.7	
SKR223	NGB101304	WBH 1304-4					18.3		
SKR224	NGB101304	WBH 1304-5					14.6		

Table 1. *Cont.*

SKR. No.	Accession	Name	AA5	AC58	AA9	TSW 2017 (g)	Protein 2017 (%)	Protein 2018 (%)	Protein 2019 (%)
SKR225	NGB101304	WBH 1304-6					19.1		
SKR226	NGB101304	WBH 1304-7				163	22.1	23.6	
SKR227	NGB101304	WBH 1304-8				154	21.4	20.9	
SKR228	NGB101304	WBH 1304-9					14.5		
SKR229	NGB101304	WBH 1304-10				102	21.3	26.5	
SKR230	NGB101836	1				123	20.9	25.1	

Of the 227 accessions grown in the greenhouse, 199 were chosen for total nitrogen analyses based on previous information of high protein content. These were grown at the experimental farm of University of Copenhagen in Taastrup (55°40′ N; 12°18′ E), Denmark in the years 2017, 2018 and 2019. One individual per accession per year was analyzed. The seeds were hand-sown at approximately 1 seed per 10 cm in a two meter single row, and nets supported the growing plants. Both greenhouse plants and plants grown in the field were organized in the order of the Nordic Genbank number. Automatic irrigation was used when needed. At maturity, whole plants were harvested keeping accessions separate and then air-dried. Pods were collected from the dry plants and threshed by hand.

2.2. Microsatellites

Total genomic DNA was extracted from green leaf material of 194 pea accessions via the CTAB method [35]. The DNA was dissolved in 50 µL 1× TE buffer and the quality of the total genomic DNA was assessed via agarose gel electrophoresis (Electrophoresis Consort EV265, Cohasset, MA, USA) and spectrophotometry on an Uvidoc (Buch & Holm, Herlev, Denmark).

The microsatellite markers AA5, AC58 and A9 were chosen after initial screening of a larger set of microsatellite markers and based on previous success reported by Loridon et al. [28]. When tested AA5, AC58 and A9 produced consistently good quality data across the large number of accessions. All forward primers had an M13-tail (CACGACGTTG-TAAAACGAC) with a dye attached. The forward primer of AA5 had a FAM, A9 a NED and AC58 a VIC dye (Table 2).

Table 2. The three microsatellite markers AA5, A9 and AC58 [28], the forward and reverse primer sequences of the markers, and the color attached to the M13-tail of the forward primer. The sequence of the M13-tail was: CACGACGTTGTAAAACGAC.

Marker	Forward Primer (5′—3′)	Reverse Primer (5′—3′)	Color
AA5	tgccaatcctgaggtattaacacc + M13	catttttgcagttgcaatttcgt	FAM, Blue
A9	gtgcagaagcatttgttcagat + M13	cccacatatatttggttggtca	NED, Yellow
AC58	Tccgcaatttggtaacactg + M13	cgtcaatttcttttatgctgag	VIC, Green

Each PCR sample consisted of 1 µL total genomic DNA, 7.25 µL double-distilled water, 1 µL 10× Dreamtaq buffer (20 mM Mg Cl_2), 0.4 µL dNTP (2.5 µM), 0.125 µL forward primer with M13-tail and color (10 µM), 0.125 µL reverse primer (10 µM), and 0.1 µL Dreamtaq Polymerase. The samples were mixed and run on a T100 Thermal Cycler (Bio-Rad Laboratories, Copenhagen, Denmark) using the following protocol: 94 °C for 4 min, 19 cycles of 94 °C for 1 min, 64 °C for 30 s (annealing), and 1 min of 72 °C. The annealing temperature was decreased half a degree for every cycle ending at 55 °C. Then, an additional 19 cycles were conducted with the same steps using a constant annealing temperature of 55 °C, followed by 10 min at 72 °C, and a final cooling to 4 °C.

The PCR products were diluted 1:5, and 3 µL of the diluted PCR product was mixed with 10 µL formamide and 0.5 µL GeneScan™ 120 LIZ™ (standard). The samples were denatured at 95 °C for 5 min and put on ice for 2 min. The samples were applied to an ABI 3130xl Genetic Analyzer (Hitachi, Tokyo, Japan) using the program Genetic Mapper Instrument protocol Fragment36_POP7_G5, and the resulting fragments were visualized on GeneScan® 3.7 Analysis Software (Applied Biosystems, Roskilde Denmark).

The resulting microsatellite band patterns were treated as binary characters where the presence of a given PCR fragment was marked as 1, absence as 0 and lacking results were marked as "?". All data from the three microsatellite markers were combined into one Microsoft Excel file and later converted to a text file (.txt) to generate the full matrix.

2.3. Thousand Seed Weight and Protein Content

Thousand seed weight (TSW) was determined by seed counting on a Contador Seed counter (Pfeuffer, Kitzingen, Germany) and then weighed.

Total N was determined via the Dumas method on a Vario Macro Cube (Elementar) [36] and crude protein content (%, *w*/*w*) was calculated using the conversion factor 5.44 [37,38].

2.4. Data Analysis

2.4.1. Polymorphism Information Content

Polymorphism information content (*PIC*) was calculated for the microsatellite data. The values were calculated using the following equation, where p_i is the frequence of the i allele [27] (see also [28]):

$$PIC = 1 - \sum p_i^2$$

2.4.2. Neighbor Joining Tree

A Neighbor Joining tree was constructed based on the binary matrix in PAUP 4.0a169 [39]. The parameters were set to BioNJ method with mean character difference as distance measure, and all characters were given equal weight. A Neighbor Joining bootstrap analysis was run with 2000 repetitions. Bootstrap values of 50% or above were manually added to the Neighbor Joining tree.

2.4.3. Principal Coordinate Analysis of Microsatellite Data

A Principal Coordinate analysis (PCoA) was performed in GenAlEx [40,41] on the microsatellite data. Binary data were converted into columns of codominant genotypic microsatellite data with loci scored as fragment size in accordance with the GenAlEx-formats. A genetic distance tri-matrix was then made in GenAlEx, from which a PCoA was performed.

2.4.4. ANOVA of Crude Protein across Years

A one-way ANOVA was performed in Microsoft Excel, comparing the difference between protein content across the three years. A Tukey–Kramer post hoc test was performed in Excel to compare the mean between each pairwise combination of years.

3. Results

Single seed descent was used to obtain a homogeneous seed stock of 227 genebank accessions for further field evaluations and for DNA extractions, molecular identification and phylogenetic analysis.

3.1. Plant Material

All seeds germinated and leaf material from all plants were collected for DNA extraction.

3.2. Microsatellites

DNAs were successfully extracted from all 194 samples selected for microsatellite analyses. PCRs from the marker AA5 resulted in 185 successful amplifications (95.4%), marker AC58 and A9 both resulted in 186 (95.8%) successful amplifications (Table 1). All together, a total of 25 accessions lacked results for one of the three primer pairs: SKR 8, 27, 42, 78, 97, 99, 100, 103, 106, 110, 117–119, 123, 125, 129, 131, 134, 135, 138, 182, 200, 202, 216 and 218 (Table 1).

The final matrix included 77 different PCR fragments from the three primer pairs. Marker AA5 provided 21 PCR fragments, marker AC58 27 PCR fragments, and marker A9 had 29 PCR fragments. The length of the PCR fragments varied from 110 to 407 base pairs (bp), where the smallest fragments were from AA5 and the largest from A9.

For the marker AA5, 5 of 21 PCR fragments were uninformative. The most common PCR fragment for AA5 was present in 64 samples and the length was 127 bp. For the marker AC58, 9 of 27 PCR fragments were uninformative. The most common in AC58 was present in 37 samples. For marker A9, 9 of 29 PCR fragments were uninformative. In A9, the most frequent PCR fragment was present in 26 accessions and had the length of 387 bp.

3.3. Thousand Seed Weight and Protein Content

Thousand seed weight (TSW) was determined for 102 field grown accessions in 2017 as a measure of seed size and ranged from 64 to 254.2 g (Table 1; Figure 1) with a mean of 152.05 g. The distribution (Figure 1) indicated that the majority of the accessions had a TSW between 120 and 180 g.

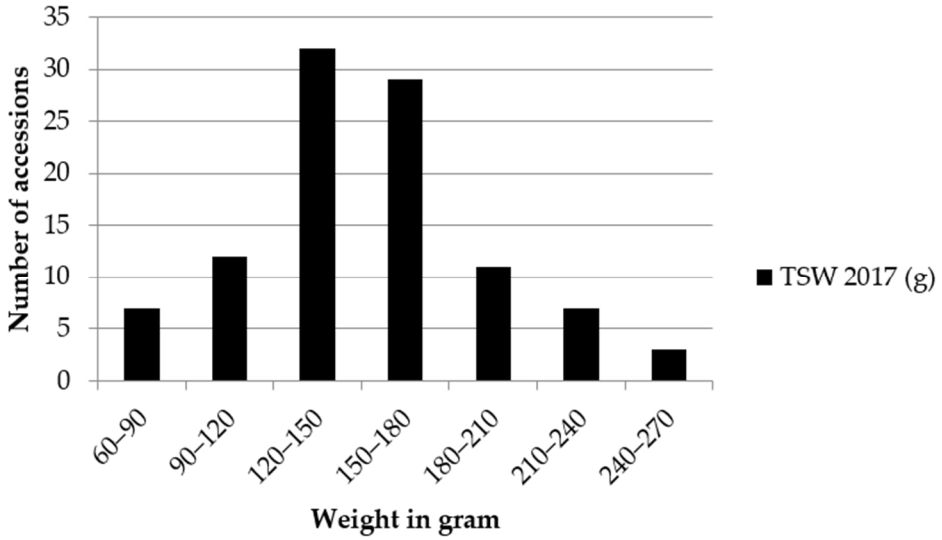

Figure 1. Thousand seed weight of pea accessions in grams (n = 102). The peas were grown with irrigation and standard agricultural management in 2017 and harvested at maturity.

The protein content was successfully determined for field grown material, with 199 accession in 2017, 95 accessions in 2018 and 30 accessions in 2019 (Table 1). The protein content varied from 9.3% (SKR 182, NGB102130) to 34.1% (SKR 64, NGB105862) for all years (Table 1) and most samples had a protein content between 16% and 28% protein content (Figure 2). All years showed a Gaussian distribution of protein content of the accessions. The average was 20.7% in 2017, 26.2% in 2018 and 22.7% in 2019, and 13 accessions had a protein content above 28% (five above 30%) in 2017, 19% (five above 30%) in 2018 and 0 in 2019. Of the 10 accessions with the highest protein content in 2017, only 1 was in the top ten in 2018, although the 95 pea accessions selected for 2018 generally had a higher protein content and were chosen among the 199 accessions with the highest protein content in 2017. One-way ANOVA revealed a highly significant difference in protein content across

the three years ($F_{2.21}$ = 66.05, $p = 9.44 \times 10^{-25}$). Tukey–Kramer post hoc test found that the protein content was significantly different among years.

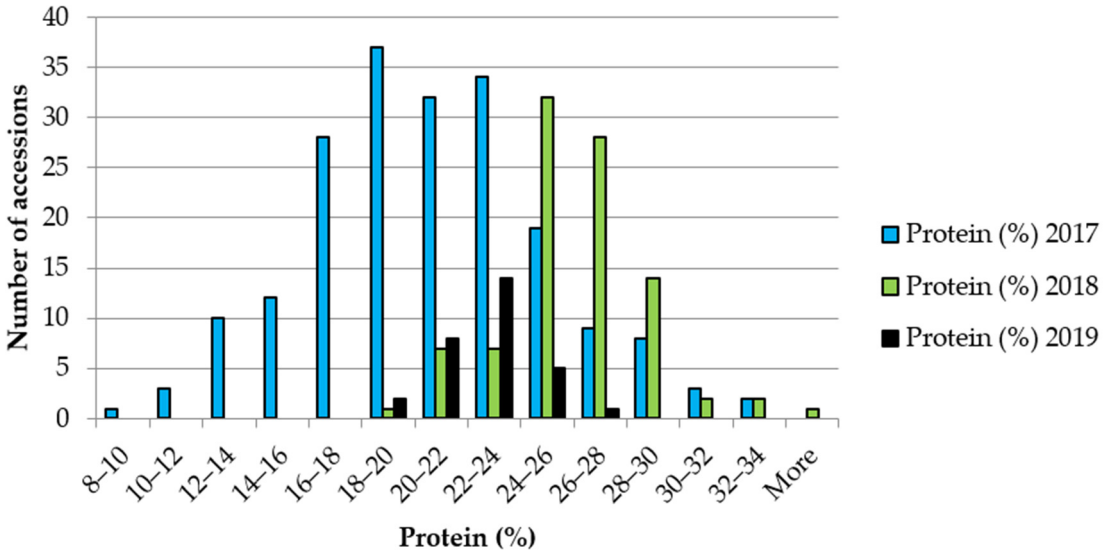

Figure 2. Protein content (%, *w*/*w*) of pea accessions. Blue represents accession were from 2017 (n = 199), Green from 2018 (n = 95) and Black 2019 (n = 30).

3.3.1. Polymorphism Information Content

The *PIC* values for marker AA5, AC58 and AA9 were 0.87, 0.90 and 0.91, respectively. The average *PIC* value was 0.89.

3.3.2. Neighbor Joining Tree

The Neighbor Joining analyses resulted in a tree topology with 107 groups of 194 accessions (Figure 3). The bootstrap analysis resulted in branch support for 11 groups ranging between 50% and 89%. Most groups did not have bootstrap support (Figure 3). We had cultivar names for 77 of the 194 accessions included in the NJ analysis. Fifty-three cultivars were represented by only one accession. Twelve accessions were from the cultivar *Pisum sativum* "chlorotica", four from "variomaculata", two from "compactum", two from "costata", two from "New Wales", and two from "Lilaska" (Figure 3; Table 1). The "chlorotica" cultivars were resolved in several different groups on the NJ tree, but most of them clustered around the same few branches (Figure 3). The other cultivars resolved far apart on the NJ tree.

3.3.3. Principal Coordinates of Microsatellite Data

The first two principal components of the PCoA accounted for 16.62% of the variance between the accessions of the three microsatellites combined (Figure 4). The pattern of variation mirrored the placement of samples in the NJ tree.

3.3.4. Nitrogen Content and Thousand Seed Weight in Relation to Genetic Diversity

The results from the total N analyses showed no apparent relationship between the protein content and the genetic diversity among the pea accessions. The accessions with high protein content did not cluster either in the NJ tree or in the PCoA.

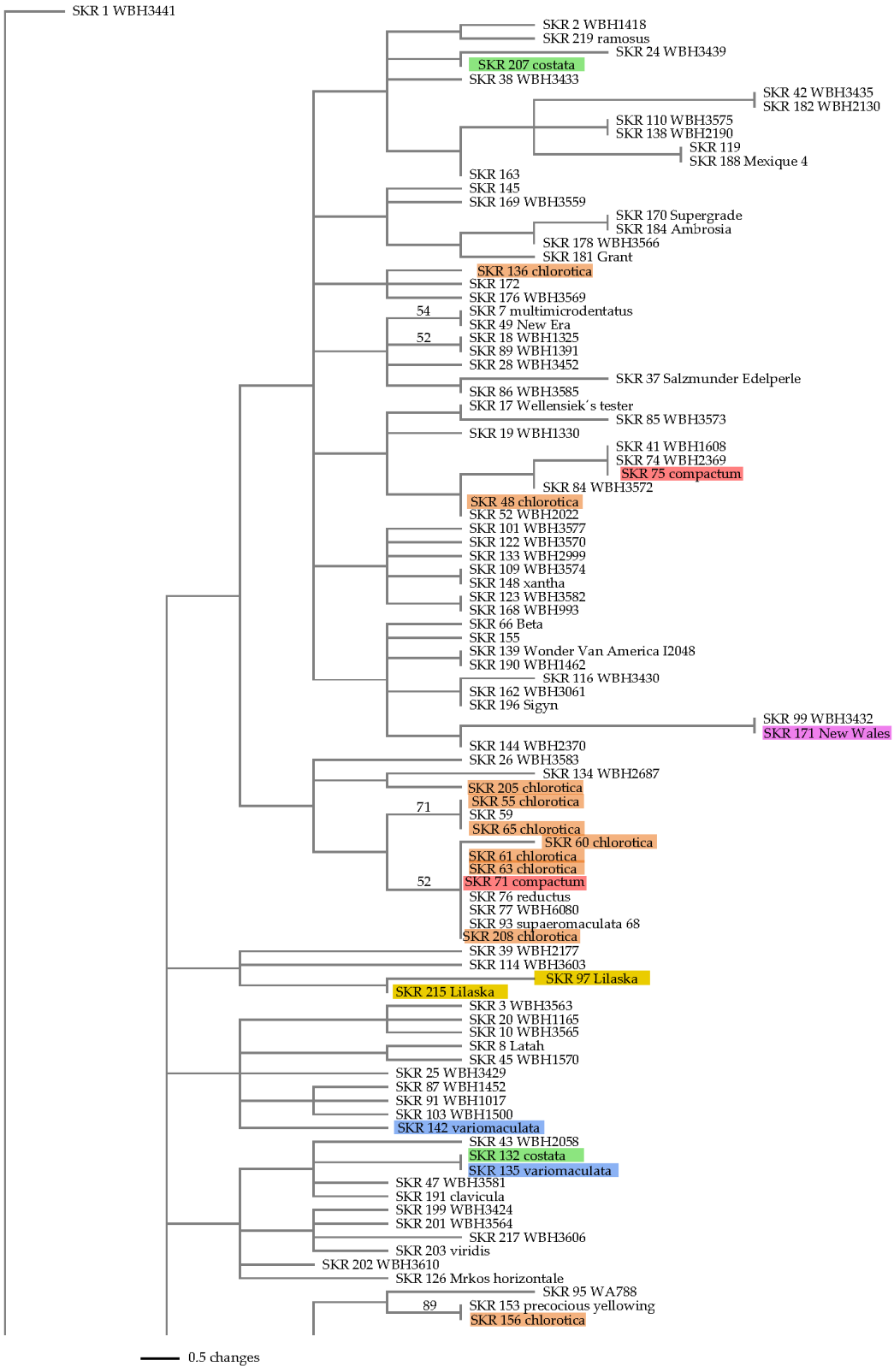

Figure 3. *Cont.*

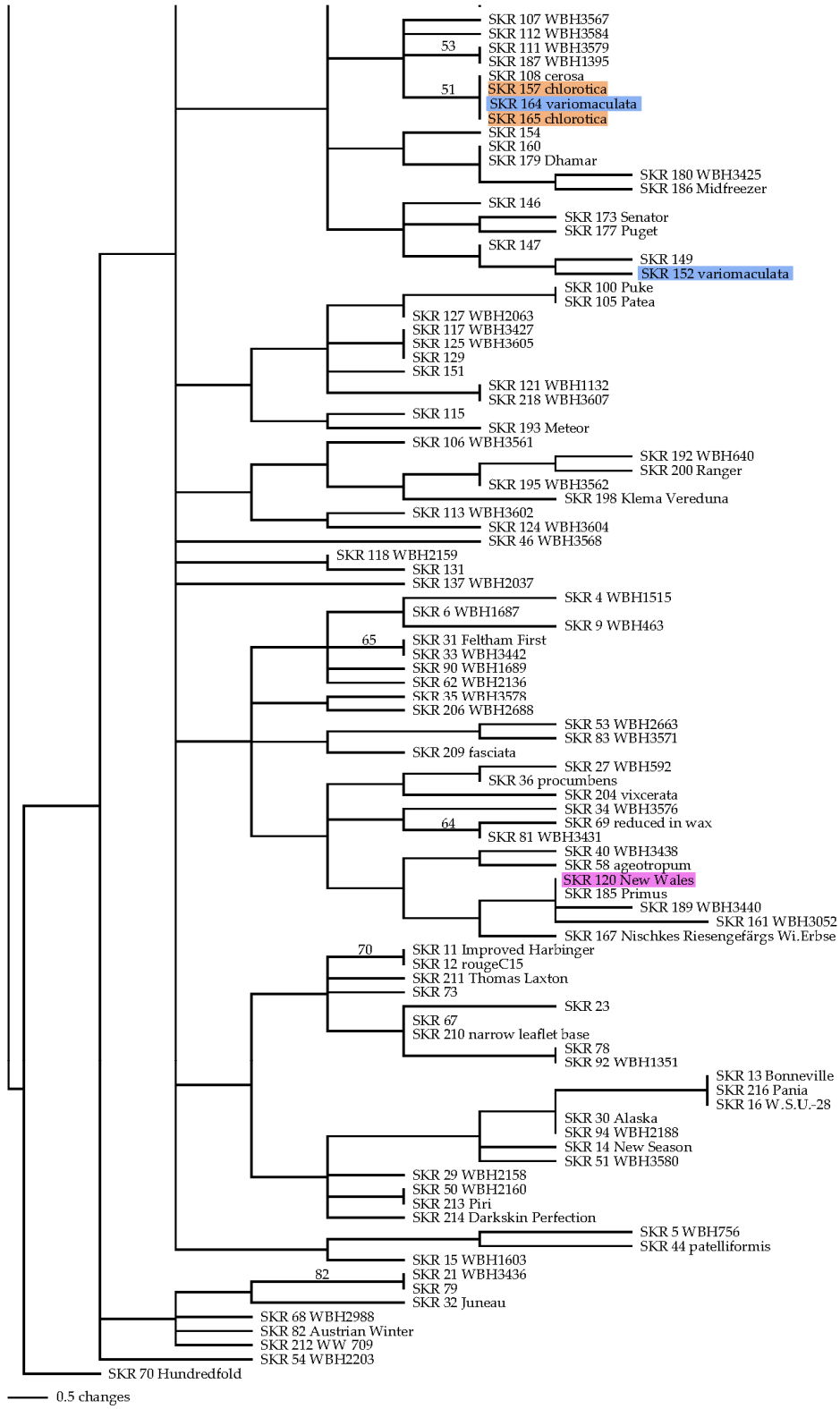

Figure 3. Neighbor Joining tree of 194 pea accessions with bootstrap values on supported branches. Highlighting indicates cultivars represented by more than one accession. Green: "costata"; orange: "chlorotica"; red: "compactum"; purple: "New Wales"; yellow: "Lilaska"; blue: "variomaculata".

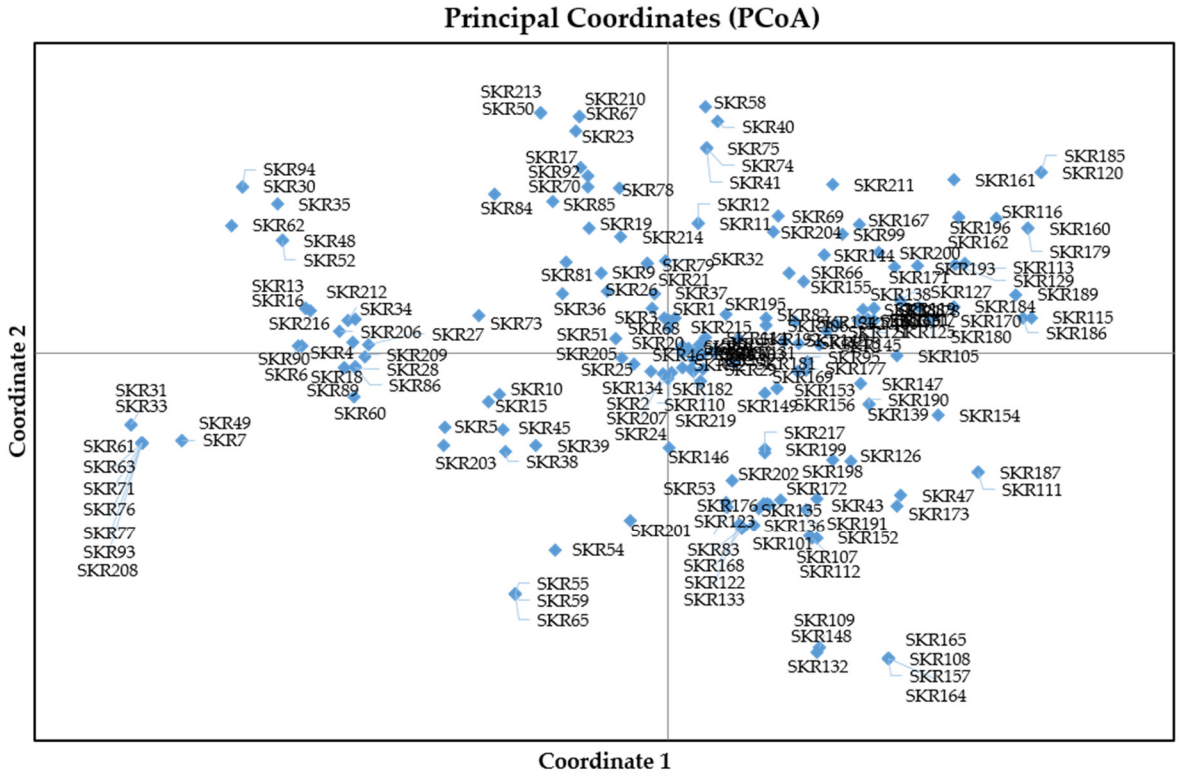

Figure 4. Principal coordinates analysis of the microsatellite results. X- and y-axis represent PC1 and PC2, respectively.

4. Discussion

The primary goal of this study was to examine the genetic diversity in pea accessions, which have primarily been grown and collected in Scandinavia. The genetic variation was investigated using three microsatellite markers, which all functioned well and resulted in high genetic variation. Furthermore, the protein content was determined for different subsets of the accessions over three years and thousand seed weight was measured for one year.

4.1. Microsatellites

The microsatellite AC58 produced 186 PCR fragments that varied between 210 and 263 bp. The number of fragments were higher than other studies where 1–11 fragments are reported, but the lengths were within what other studies have found: 200–263 bp [15,19,28,31,34]. The microsatellite AA5 produced 185 different PCR fragments of lengths from 110 to 264 bp. The number of fragments were higher than other studies where 1–3 fragments are reported, and the lengths spanned a wider range than the 225 to 250 bp reported in other studies find [15,28,34]. The microsatellite A9 produced 186 different PCR fragments with lengths that varied between 325 and 407 bp. The number of fragments were higher than in other studies, where one to six fragments were reported, and the lengths spanned a somewhat wider range than the 330–390 bp observed in previous studies [14,17,18,21,28,31].

To sum up, most previous studies produced 2–4 DNA fragments for each microsatellite, except for the study of Hagenblad et al. [19], where 14–33 DNA fragments were found. Hagenblad et al. [19], like in our study, used Scandinavian pea accessions from NordGen. This indicates that the collection of Scandinavian pea accessions at NordGen are genetically diverse. The results could have been even more discriminating if more than three microsatellites had been analyzed.

4.2. Polymorphism Information Content

The *PIC* values in this study (0.87–0.91) were high when compared to other studies. The reported *PIC* values for pea accessions fall within a wide range from 0.3 to 1.0 for individual microsatellite markers. The variation in *PIC* values is higher in studies developing microsatellites, e.g., *PIC*s varied between 0.04 and 1.0 with an average of 0.63 in Loridon et al. [28]. Many studies focus on the genetic diversity within a specific group of accessions and use already tested microsatellites. The *PIC* values of these studies do not span the same variation as Loridon et al. [28] did. A few studies did find *PIC* values as high as in this study. Burstin et al. [13] use a large number of accessions and many microsatellites and obtained *PIC* values between 0.46 and 0.97 with a mean of 0.8 for 372 wild and cultivated pea accessions using 29 microsatellites. Nasiri et al. [33] found an average *PIC* value of 0.72 for 77 pea accessions from 17 different countries with 10 microsatellites. However, most studies found lower *PIC* values than our study, between 0.3 and 0.65, most using a larger number of microsatellites [14,16–18,20–24,28,30–32]. Singh et al. [20] use 20 microsatellites to assess the genetic diversity of 47 pea accessions from India and obtained an average *PIC* value of 0.55, ranging from 0.04 to 0.85. Another study [22] assessed 40 pea accessions with five microsatellites and obtained *PIC* values between 0.14 to 0.82. Of all studies assessing genetic variation in peas [13–26,29–34], only three studies [13,15,33] reported results where all the microsatellites had a *PIC* value of 0.46 or higher.

The *PIC* values are mainly affected by the variation among the included individuals; however, the number of microsatellites may affect the outcome of the average *PIC* value because the number affects the statistical strength by potentially adding more variation [27,31]. The genome of *Pisum sativum* is large, approximately 4.45 giga base pairs [3], and therefore the results of this study could be affected by the low number of microsatellites included three.

The *PIC* values in this project varied between 0.87 and 0.91 with an average of 0.89. For the same specific microsatellites, Loridon et al. [28] finds *PIC* values of 0.69 (A9), 0.84 (AC58) and 0.78 (AA5). Other studies using the A9, AC58 and AA5 microsatellites find *PIC* values between 0.61 and 0.78 [14,15,17–19,31,34] except for the A9 microsatellite of Haliloglu et al. [17], which has a low *PIC* value of 0.03.

The *PIC* values of this study were therefore among the highest reported for genetic variation among pea accessions [13–26,28–34]. The average *PIC* value is not only affected by the variation among individuals for each microsatellite included but also by the number of accessions, because the chance of identifying different alleles rises with the number of accessions included [27,31]. Thus, the higher *PIC* values in the present study could partially be explained by the larger number of accessions (194) compared to most studies [16–23,26,28,29,31–34]. Some studies included more than 100 accessions [13–15], however, and few more than 500 [24,25,30].

This study, along with most of the studies on genetic diversity of peas, placed the microsatellites used in the category, "highly informative", because the *PIC* values are above 0.5 [27]. The high *PIC* values of this and other studies could be explained by the selection of the most polymorphic markers from the study of Loridon et al. [28] or similar studies where a large number of microsatellites were included. The high average *PIC* value of this study could be interpreted as a reflection of a high genetic variation in general among the 194 accessions included. This high genetic variation indicates that the accessions had not undergone a targeted breeding for specific agronomical traits and homozygosity [32]. The high *PIC* values might thus be explained by the inclusion of a large number of pea accessions with different breeding histories.

4.3. Neighbor Joining Tree

The Neighbor Joining tree uniquely identified 153 of the 194 accessions (78%) and 108 groups were resolved as monophyletic using three microsatellites (Figure 3). Jain et al. [32] uniquely identify all 96 individuals included in the study in the NJ topology, but they included 42 polymorphic markers, distributed throughout the genome. Many

studies using more microsatellites have produced a full resolution of the NJ tree [26,31,32]. One study using three microsatellites obtained 95% resolution of the topology, but this study was partly at the species level [29], whereas the present study was below the species level. In the study by Bouhadida et al. [30], 18 of 19 accessions (94.7%) are uniquely identified on an unweighted pair group method (UPGMA) topology through the use of five microsatellite markers. The UPGMA and NJ methods use the same underlying model, but UPGMA assumes a similar rate of evolution rate along the branches of the tree (molecular clock) [42]. The bootstrap support values of our study were low (Figure 3) [43]. The low bootstrap support and the lack of resolution in the NJ topology is mainly explained by a lack of variation due to insufficient DNA fragments of each microsatellite. A total of 77 variable DNA fragments is not enough to fully resolve 194 accessions [43]. Another possible explanation for the low bootstrap support is the taxonomic level. For studies below the species level, bootstrap values cannot always be expected to be high [43]. A third factor affecting the outcome of the NJ topology in this study is missing results for some individuals. Twenty-five accessions lacked data for one of the three microsatellites, which contributed to greater uncertainty in the branching pattern and collapse of branches [44].

The genetic diversity of named cultivars was expected to be structured such that accessions with the same cultivar name would be included in the same monophyletic group. However, such a structure did not appear. The 12 cultivars for which we had more than one accession were resolved in different positions on the NJ tree (Figure 3, colored cultivars). One group included accessions from "chlorotica", "reductus", "superaeromaculata" and "compactum" (Figure 3, colored cultivars). This lack of structure could partly be explained by the lack of sufficient data [3,24,34]. However, the resolution of individuals from the same cultivar at different places in the NJ tree may also be the result of genetic variation among individuals. This variation may be due to separation in time creating genetic diversity because of crosspollination resulting in polymorphism [29]. Peas from Ethiopia are genetically similar and resolve as a monophyletic group, whereas accessions from other countries, e.g., USA and Norway, do not show such similarity and do not resolve as monophyletic [33]. This lack of similarity can be explained by a gene flow between countries, e.g., use of genetic material from more than one country. The lack of a clear pattern among cultivars included in this study might be due to a high exchange of genetic material among breeding companies in Scandinavia, because the accessions of the same cultivar were not necessarily from the same source.

4.4. Thousand Seed Weight and Protein Content

Seed size is an important agronomic trait for yield and productivity that is inherited and characteristic of specific cultivars. Seed size is a quantitative trait controlled by many genes. Thousand seed weight is a simple method for measuring seed size. We found large variation from 64.4 g (SKR 46, NGB103568) to 251.2 g (SKR 218, NGB103607).

Protein content varied from 9.3% (SKR 182, NGB102130) to 34.1% (SKR 64 NGB105862) over all years and accessions. One goal of this study was to identify high-protein pea accessions, and 13 were found to have a protein content above 28% in 2017 and 19 in 2018, but none in 2019. This emphasizes the variation in protein content across the years, as the accessions with determined protein content for the years following 2017 was selected for high protein content. The selection of peas with a high protein in 2017 for analyses in 2018 moved the distribution to the right (Figure 2). We expected the protein content of the 2019 samples to show the same pattern and be placed even further to the right, but this was not the case. Year to year variation in protein content is well documented and is assumed to be due to differences in weather [45]. In comparison to other studies, the protein content was 23.9 $\pm$ 2.5% and TSW 195.9 $\pm$ 16.9 g for a collection of 1222 accession of cultivars and landraces cultivated during 1979–1982 with irrigation and standard management in Sweden [46]. A recent study of 50 accessions assessed for their potential in the Arctic region showed an average protein content of 26% in Denmark, 24% in Sweden and 20% in Finland [47].

An analysis of 198 peas (*Pisum sativum* L. cv. Trapper) from Saskatchewan farmers showed a protein content of 14.5% to 28.5% with a Gaussian distribution [11]. The protein content was determined from the Kjel-Foss method with a conversion factor of 6.25. Wang and Daun [10] determined the protein content via the Dumas method (N × 6.25) for six samples of four different pea varieties with a crude protein content ranging from 20.2% to 26.7%. Thus, the studies show that there is natural variation among peas. The variation in our study was more wide-ranging, and while some accessions had a lower protein content than that reported in previous studies, there were accessions that were higher than in most other reports. Only Slinkard [48] reported a protein content above 30%.

4.5. Future Perspectives

The next step could be to investigate the history of and characterize the cultivars and accessions included in this study. These accessions were primarily from Scandinavia and all from Nordic Gene bank and they were chosen primarily because there were some indications but not necessarily confirmations of high protein content.

A high genetic diversity among pea accessions from a gene bank is valuable because researchers want to preserve as much diversity as possible for future breeding. By targeted crossing of genetically diverse individuals, a selection of plants with desirable traits such as high protein, high resistance or tolerance to drought, followed by breeding towards homozygosity, it is possible to actively use the preserved genetic diversity to develop pea cultivars for the future [3,12,32].

Microsatellites are only one of several methods to study genetic diversity. Microsatellites are popular due to their co-dominance and high polymorphism. Methods in which differences across the genome are targeted give more data and are more likely to resolve genetic diversity among accessions. An example of such a method is Diversity Array Technology (DArT), [49].

Our results increase our understanding of genetic diversity across pea varieties, and could contribute to the sustainable cultivation of peas and adaptation to climate change.

5. Conclusions

Homogeneous lines of 227 pea accessions from seed stocks at NordGen were produced via single seed descent. The peas were grown in a field for 2 years and the seed protein content showed a variation between 9.3% and 34.1% over years and accessions and thousand seed weight were on average 152.05 g. More than 10 accessions had a protein content above 28%, showing that the collection has potential as breeding nursery for high-protein pea cultivars. Three microsatellites were highly informative. The microsatellites separated 153 of 194 accessions and had *PIC* values between 0.87 and 0.91, indicating that the gene bank material contains a large number of pea accessions with different breeding.

Author Contributions: Conceptualization, S.K.R., G.P. and C.B.A.L.; methodology, S.K.R. and C.B.A.L.; software, L.W. and C.B.A.L.; validation, C.B.A.L. and L.W.; formal analysis, C.B.A.L., L.W. and S.K.R.; investigation, C.B.A.L., S.K.R. and L.W.; data curation, L.W. and C.B.A.L.; writing—original draft preparation, L.W.; writing—review and editing, L.W., S.K.R. and C.B.A.L.; visualization, L.W. and C.B.A.L.; supervision, C.B.A.L. and S.K.R.; project administration, S.K.R.; funding acquisition, S.K.R. All authors have read and agreed to the published version of the manuscript.

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