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ORIGINAL ARTICLE

Genetic markers and morphometric analysis reveal past hybridization and introgression in putative *Carex flava* L. s.str. (Cyperaceae) hybrid populations

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Abstract The affinities of taxonomically problematic populations are typically analyzed using molecular markers. However these are less subject to selection than morphological characteristics. Consequently both approaches may be required to obtain a fuller picture of the identity and history of populations. Three putative hybrid populations within the *Carex flava* agg. are examined using such a dual approach with a view to elucidate their taxonomic affinities. Analysis of 11 morphometric characters using principal component analysis and examination of 17 isozyme loci revealed a more complicated history than that suggested by morphometric analysis alone. Results from this study confirm the status of an additional British population of *C. flava* s.str. and also strongly suggest that a population morphologically resembling *Carex lepidocarpa* has experienced the introgression of *C. flava* genes in the past. An Irish population resembling *C. flava* in appearance but lacking typical *C. flava* allozymes may be a result of local selection.

Keywords Agglomerative hierarchical clustering · Allozymes · *Carex* · Cyperaceae · Introgression · Hybridization · PCA · Sedges

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Introduction

Hybridization in vascular plants is considered a common phenomenon (Ellstrand et al. 1996; Mallett 2005) and of considerable evolutionary importance (Barton and Hewitt 1989; Barton 2001). Consequently hybrid populations are of particular interest, although the recognition of such populations may be problematic (Hardig et al. 2000). The traditional approach has been the generation of hybrid indices based upon morphological characteristics, with hybridization detected by morphological intermediacy (e.g. Randolph et al. 1967; Potter 1994; Diskin et al. 2006). However such an approach can be difficult when a large degree of overlap is present in several of the potentially discriminating characteristics. The measurement of continuous characters allied to multivariate ordination techniques has been successfully employed to resolve these problems (e.g. Tauleigne-Gomes and Lefèbvre 2005; Dean and Ashton 2006). However Rieseberg and Ellstrand (1993) and Rieseberg (1995) demonstrated that hybridization does not always lead to morphological intermediacy, especially when introgression is extensive and/or the hybridization event is historic. Moreover multivariate ordination techniques alone cannot differentiate between divergence and hybridization (Wilson 1992). This poses particular problems for identifying hybrid populations when one of the putative parents is no longer extant at the site.

As a result of these drawbacks, the majority of hybridization studies over the last 30 years have utilised molecular markers. However Reed and Frankham (2001) in an extensive meta-analysis showed that there is little correlation between the results from molecular data and those from quantitative measures. This is unsurprising, given that quantitative characteristics are under polygenic control

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whose expression is influenced by the environment. In addition they are likely to be influenced by selection (Christensen 2005; Bolvansky and Uzik 2005). By comparison molecular markers are under simple genetic control, uninfluenced by the environment and are generally considered to be selectively neutral and thus more likely to be influenced by factors such as genetic drift and gene flow (Lowe et al. 2004). Such contrasting forces acting on the two types of characteristics suggest a more informative picture on the nature and evolutionary history of taxonomically complex populations may be obtained by considering molecular and morphological results together. This study seeks to use such a combined morphometric and genetic approach to identify the historic hybridization or otherwise of three putative *Carex flava* L. × *Carex lepidocarpa* Tausch. (=*C.* × *pieperana* P. Junge) hybrid populations within the British Isles.

C. flava s.l. is a taxonomically complex group of plants with several recognized taxa that has attracted considerable attention. There is general agreement based upon morphology (Crins 1985; Crins and Ball 1989a; Davies 1953; Schmid 1980) or allozymes (Bruederle and Jensen 1991; Hedrén 2002) that *C. flava* and *C. lepidocarpa* are distinct taxa, although the allocation of rank has been subject to debate. Davies (1953) treated both of the two taxa as separate species, while Schmid (1980) and Crins and Ball (1989b) considered *C. flava* to be a species but only recognised *C. lepidocarpa* as a subspecies of *Carex viridula*. By contrast Sell and Murrell (1996) considered both to be subspecies of *C. flava* s.l. Recent studies utilizing a combination of morphometric and allozyme analysis support the recognition of *C. lepidocarpa* at the specific level and separate from *C. viridula* (Hedrén 2002; Blackstock 2007). To some degree the level of taxonomic separation depends upon the species concept adopted by the various authors. Perhaps more significant is that the systematic relationship of the two taxa is under less debate with *C. flava* s.str. being considered a sister group to the clade which contains *C. lepidocarpa* (Hedrén 2004).

In Britain *C. lepidocarpa* is a common species typically found in damp unshaded sites with alkali soils. Globally it has an amphiatlantic distribution, being widespread in Europe and restricted to the eastern seaboard of North America (Crins 1985). *C. flava* s.str. has a wider distribution than *C. lepidocarpa* being circumboreal. However it has always been a very rare plant in the British Isles having only ever been recognised from four localities, two of which are now extinct. It is currently categorised as vulnerable in Britain (Cheffings and Farrell 2005), with the largest extant population in the British Isles being found at Roudsea Wood, Haverthwaite, Cumbria (v.c. 69). A second *C. flava* type population is also known from Malham Tarn Moss (v.c. 64). However the identification of this population has been subject to some doubt as the plants appear to be significantly smaller than those found at Roudsea Wood. Jermy and Tutin (1968) speculated that this population was probably a hybrid, *C.* × *pieperana* with *C. lepidocarpa* as the other parent. This view prevailed amongst British botanists until an extensive morphometric analysis (Blackstock and Ashton 2001) indicated that this population was indeed *C. flava* s.str. Two further putative hybrid populations of *C. flava* in the British Isles have been described: Greywell Moors, Hants (v.c. 12; Brewis et al. 1996) and Coolagh Fen, Lough Corrib, Co. Galway (v.c. H17; Perring 1970). In both cases *C. flava* s.str. is no longer considered to be extant at these sites, having been ousted by introgression with *C. lepidocarpa* (Jermy et al. 1982). These populations were not examined by Blackstock and Ashton (2001) due to a lack of material from these sites at that time.

Although morphometric analysis of the Malham Tarn Moss populations strongly supports the recognition of this population as *C. flava* s.str. it is desirable, given the morphological similarities within the group, that molecular markers, such as allozymes, combined with multivariate statistical analyses (Lowe et al. 2004) are used to provide an additional line of evidence to support this conclusion. Additionally the populations at Coolagh Fen and Greywell Moors also need to be assessed using the same approach. Allozymes have been used to successfully elucidate systematic relationships within the genus *Carex* (Bruederle and Fairbrothers 1986; McClintock and Waterway 1993; Ford et al. 1991) and specifically within the *C. flava* agg. (Bruederle and Jensen 1991; Hedrén 1996; Hedrén and Prentice 1996; Hedrén 2002). This study will seek to (1) identify morphological differences between *C. flava* and *C. lepidocarpa*, (2) identify genetic markers that differentiate *C. flava* from *C. lepidocarpa*, and (3) utilise both sets of markers to address the identity and history of the putative hybrid populations.

Materials and methods

Sources of material

Material was collected from 36 sites in North America, Europe and the British Isles (Table 1). The aim was to provide material from across the range of the species as far as was practical. Populations included 13 *C. flava*, 20 *C. lepidocarpa* and the three putative hybrid sites, Malham Tarn Moss, Greywell Moors and Coolagh Fen. All *C. lepidocarpa* material was of ssp. *lepidocarpa*.

Specimens of *C. flava* from Canada provide a valuable comparison, as these populations are approximately 700 km from the nearest *C. lepidocarpa* population. Some

Table 1 Localities of the populations of yellow sedges (*Carex flava* agg.) included in the morphometric (M) and allozyme (A) analysis

Location	Country	Location	Number of individuals sampled	Taxon
Rosentrop, Hälsingland[a]	Sweden	61°23′N	47 (A)	*C. flava*
		14°49′E	2 (M)	
Dalbyn, Ore, Dalarna	Sweden	61°08′N	38 (A)	*C. flava*
		15°10′E	9 (M)	
Västanå, Boda, Dalarna	Sweden	60°59′N	34 (A)	*C. flava*
		15°12′E	7 (M)	
Kuusamo	Finland	65°58′N	7 (A)	*C. flava*
		29°10′E		
Suomussalmi	Finland	64°54′N	7 (A)	*C. flava*
		29°02′E		
Vågå, Lemonsjøen	Norway	61°46′N	11 (A)	*C. flava*
		09°04′E	9 (M)	
Nes, S. Rakeie	Norway	60°01′N	14 (A)	*C. flava*
		11°45′E		
Champigneulles	France	48°44′28″N	15 (A)	*C. flava*
		06°08′40″E		
Roudsea Wood, Cumbria	England	54°13′36″N	48 (A)	*C. flava*
		03°01′34″W	6 (M)	
Nr. Orangeville, Peel Co., Ontario	Canada	43°53′N	20 (A)	*C. flava*
		79°59′W	1 (M)	
Snowdons Corners, Grenville Co., Ontario	Canada	44°49′43″N	49 (A)	*C. flava*
		75°46′12″W	1 (M)	
Sundew Bog, British Columbia	Canada	49°53′N	16 (A)	*C. flava*
		125°37′W	3 (M)	
Campbellville, Halton Co., Ontario	Canada	43°31′N	15 (A)	*C. flava*
		79°59′W	2 (M)	
Malham Tarn Fen, Yorkshire	England	54°06′N	15 (A)	*C. flava* or hybrid?
		02°11′W	10 (M)	
Greywell Moors, Hants	England	51°15′N	16 (A)	Hybrid?
		00°58′W	11 (M)	
Coolagh Fen, Lough Corrib, Galway	Ireland	53°18′N	34 (A)	Hybrid?
		09°03′W	6 (M)	
Lyngsjön, Lyngsjö, Skåne[b]	Sweden	55°55′50″N	22 (A)	*C. lepidocarpa*
		14°04′06″E	4 (M)	
Benestad, Örups kärr, Skåne[b]	Sweden	55°30′52″N	32 (A)	*C. lepidocarpa*
		13°55′34″E		
Örbacken, Allhelgona, Östergötland[b]	Sweden	58°22′20″N	30 (A)	*C. lepidocarpa*
		15°07′06″E		
Benestad, Benestads backar, Skåne[b]	Sweden	55°31′44″N	29 (A)	*C. lepidocarpa*
		13°53′47″E	4 (M)	
Sjöstorp, Ödeshög, Östergötland[b]	Sweden	58°15′N	24 (A)	*C. lepidocarpa*
		14°39′E		
Mörka hål, V. Tollstad, Östergötland[b]	Sweden	58°19′N	46 (A)	*C. lepidocarpa*
		14°39′E		
Hiiumaa Island	Estonia	59°10′N	2 (M)	*C. lepidocarpa*
		22°30′E		
Prission, S-Tirol	Austria	47°N	2 (M)	*C. lepidocarpa*
		12°E		

Table 1 continued

Location	Country	Location	Number of individuals sampled	Taxon
Locheres	France	47°41′N 04°55′E	1 (M)	*C. lepidocarpa*
Vaux-les-Palameix	France	49°00′N 05°31′E	42 (A)	*C. lepidocarpa*
Hawes Water, Lancashire	England	54°11′N 02°48′E	6 (M)	*C. lepidocarpa*
Crummack Farm, Inglebrough, North Yorkshire	England	54°08′N 02°21′E	5 (M)	*C. lepidocarpa*
Malham Tarn Outflow, North Yorkshire	England	54°06′N 02°11′E	6 (M)	*C. lepidocarpa*
Woodbastwick, Norfolk	England	52°41′N 01°27′E	3 (M)	*C. lepidocarpa*
Scarning Fen, Norfolk	England	52°40′N 00°53′E	14 (A)	*C. lepidocarpa*
White Coppice, Lancashire	England	53°40′N 02°34′W	36 (A)	*C. lepidocarpa*
Glen Fender, Perth and Kinross	Scotland	56°34′N 04°05′E	5 (M)	*C. lepidocarpa*
Fearnan, Loch Tay, Perth and Kinross	Scotland	56°47′N 03°48′E	1 (M)	*C. lepidocarpa*
Carran An Carn, Co. Clare	Ireland	53°002′N 09°03′W	30 (A)	*C. lepidocarpa*
Rinnamona Lough, Co. Clare	Ireland	53°02′N 09°03′W	24 (A) 1 (M)	*C. lepidocarpa*

[a] Included in Bruederle and Jensen (1991)

[b] Used by Hedrén and Prentice (1996)

sites were also sampled in work by Hedrén and Prentice (1996) or Bruederle and Jensen (1991) (see Table 1). The inclusion of these sites confirms the identification of the taxa but differences in methodology preclude direct comparison of allozyme mobility. Material was collected as either ramets or seeds from clumps grown at least 3 m apart. Members of the *C. flava* agg. have a caespitose growth habit, so each sample was presumed to be an individual genet. Material collected was maintained as separate genets, except for the North American material which was bulked as seed. The individual specimens were cultivated under similar conditions at Edge Hill University for 1 to 2 years until the plants had reached maturity. A total of 715 specimens were used in the allozyme analysis and 107 individuals were used in the morphometric analysis. Not all specimens were subject to both forms of analysis due to loss of plants between the different stages required for the two processes, with allozymes requiring young tissue and morphometrics utilizing mature plants.

Morphometric analysis

The methods of morphometric analysis outlined in Blackstock and Ashton (2001) have been employed here. All morphometric data were collected from mature living material, with the exception of the two putative *C.* × *pieperana* hybrid voucher specimens (BM) collected by F. Rose from Greywell Moors, 1978, and F. H. Perring from Coolagh Fen, 1968. An initial sampling of 20 variables (Table 2) essentially provided a preliminary assessment of previous classifications and an indication of the most taxonomically useful characters. Characters with very low loadings along PC I, PC II and PC III were considered to make minimal contribution to the delimitation of the species and were excluded from the main analysis. From this initial data set a second reduced number of 11 variables were then used for the multivariate analysis of the morphological characters.

Previous morphometric studies of the *C. flava* agg. (Schmid 1980; Crins 1985; Stoeva and Štepánková 1990;

Table 2 Full list of morphometric characters with their abbreviations measured from living plant specimens of the *Carex flava* agg. plus component loadings of characters used in final analysis

Variable	Abbreviation	Used in initial assessment	PC I loading in final assessment	PC II loading in final assessment
Veg. leaf length/width	VEGLEAF	X	0.206	0.265
Veg. leaf width at base (mm)	VEGBAS	X	0.285	−0.147
Veg. leaf width 5 mm beneath apex (mm)	VEGAPEX	X		
Ligule length	LIGL	X	0.348	−0.201
Culm height to uppermost female spike (mm)	CULM	X		
Culm width at base (mm)	CULMBAS	X		
Male spike length (mm)	MSPIKE	X	−0.321	−0.054
Male spike peduncle length (mm)	MPEDL	X	−0.377	−0.082
Number female spikes	FEMNO	X		
Utricle body length (mm)	PERBOD	X		
Beak length (mm)	BEAKL	X	0.399	0.124
Utricle length (mm)	PERLE	X	0.418	0.063
Beak curvature (°)	BEAKCUR	X		
Utricle width (mm)	PERLEWID	X		
Male glume length/width	MGLUM	X	0.151	−0.434
Female glume length/width	FGLUM	X	0.082	0.775
Number bristles on one side of perigynia	BRISTLE	X	0.380	−0.145
Nut length excluding beak (mm)	NUTL	X	0.054	−0.169
Nut width (mm)	NUTWID	X		
Nut beak length (mm)	NUTBEAK	X		

PERLE, BEAKL and BRISTLE have the highest components loadings along PC I. FGLUM, MGLUM and VEGLEAF have the highest loadings on PC II

Pykälä and Toivonen 1994; Hedrén 2002) have used a wider range of characteristics associated with the position of the female spikelets. Heide (2004) demonstrated that these characteristics may not be directly comparable, as ecological variables such as light levels and temperature could alter the sex expression of flowers within *C. flava*. This could result in male spikelets possessing female flowers or even becoming entirely female spikelets. Consequently they have not been included in this study. Hedrén (2002) utilized characteristics associated with spikelet number, such as the length of the associated bract. As the number of spikelets varies this would have incorporated a large number of missing data sets. Therefore he excluded such characters from his multivariate analyses, and they have likewise have not been incorporated in this study. Crins (1985) and Rothrock et al. (1997) noted that perigynia from the lower parts of the pistillate spikelets were more variable than those from the middle of the spikelet. Therefore the characters beak length (BEAKL), utricle length (PERLE) and the number of bristles on the side of the perigynia (BRISTLE) have all been taken from perigynia collected from the middle of the pistillate spikelet.

All 11 characters were then used in PCA to summarise variation patterns within the taxa covered. Data were standardised for PCA so that each variable would have a mean of zero and a standard deviation of one, and analysis was carried out using the XLStat (Addinsoft 2005).

Allozyme analysis

Fresh soluble enzyme extracts were prepared each morning, with each gel including a sample freshly extracted from a reference *C. flava* plant collected from Roudsea Wood. Approximately 1 cm^2 of vegetative material was removed from the mid section of a young leaf and placed into a pre-chilled, round bottomed microtitre plate well containing a small amount of washed sea sand to facilitate cell breakage. The sample was homogenised, using a glass rod, in 0.25 ml of an extraction buffer consisting of 50 ml gel buffer, 0.25 ml Triton-X100, 10 ml PVP 40T and 0.05 ml 2-mercaptoethanol. The crude protein extract was then absorbed onto a sample wick (3 × 8 mm) cut from Whatman no. 5 filter paper. Starch gel electrophoresis of allozymes employed 11% gels prepared using one of three buffer systems.

Enzyme staining followed standard procedures (cf. Wendel 1990 with minor modifications available from one of the authors, N.B., on request). Eleven enzyme systems with 17 putative loci were analysed. Loci are numbered sequentially from the most cathodal

locus and individual alleles coded with lowercase letters, beginning with the most cathodal allele. Two loci of PGI: E. C. 5.3.1.9, two loci of PGM: E. C. 5.4.2.2, three loci of MDH: E. C. 1.1.1.37, two loci of ME: E. C. 1.1.1.40 and one locus of DIA: E. C. 1.6.99 were resolved using a discontinuous histidine-HCl system, 0.02 M L-histidine HCl titrated to pH 7.0 with NaOH gel buffer and 0.4 M trisodium citrate titrated to pH 7.0 with HCl electrode buffer (Gottlieb 1981). Gels were run at 50 mA in a cold room set at 4°C until the bromophenol blue marker front had migrated anodally 8–10 cm, approximately 3 h. One locus of 6PGD: E. C. 1. 1. 1. 44, one locus of SKD: E. C. 1.1.1.25, one locus of GDH: E. C. 1.4.1.2 and two loci of MNR: E. C. 1.6.99.2 were resolved using a Tris-citrate system, 35 ml of electrode buffer; 0.223 M Tris/0.086 M citric acid monohydrate titrated to pH 7.5 with NaOH was diluted to 1 l to form the gel buffer (Soltis et al. 1983). Gels were electrophoresed at 50 mA for approximately 3 h. ADH: E. C. 1.1.1.1 and TPI: E. C. 5.3.1.1 were resolved using a discontinuous lithium-borate system; the electrode buffer consisted of 0.029 M lithium hydroxide/0.192 M boric acid adjusted to pH 8.3 using sodium hydroxide pearls. The gel buffer consisted of nine parts of a second buffer, 0.007 M citric acid and 0.05 M Tris base to one part electrode buffer (Ashton 1990). Gels were run at 70 mA for approximately 3 h.

Allele frequencies within each species were calculated from unweighted population averages. Population allele frequencies were collected for each locus using Popgene version 1.31 (Yeh and Boyle 1997) and used to calculate Nei's unbiased genetic identity (Nei 1978) between all populations and species. A phenogram based upon Nei's genetic identity was then constructed using XL Stat (Addinsoft 2005).

Results

Morphometric analysis

The first two principal components account for 45.39% of the variation within the data set with PC I accounting for 32.13% of the variation and PC II accounting for 13.26%. Perigynium features PERLE, BEAKL and BRISTLE had the highest component loadings (Table 2) along PC I whereas female glume length-to-width ratio (FGLUM), male glume length-to-width ratio (MGLUM) and vegetative leaf length/width (VEGLEAF) had the highest component loadings along PC II. Hence separation along both axes is related to size.

Scatter plots of PC I versus PC II (Fig. 1) reveal two clusters separated along PC I although separation along this axis is minimal. These clusters represent a *C. lepidocarpa* and a *C. flava* cluster. Within the *C. lepidocarpa* cluster falls all of the *C. lepidocarpa* plants and those putative hybrids taken from Greywell Moors, including the voucher specimen collected by F. Rose. The *C. flava* cluster incorporates all material ascribed as *C. flava* as well all putative hybrid specimens from Malham Tarn Moss and Coolagh Fen although there is partial separation along PC II for the latter. No putative hybrids are found intermediate between the *C. flava* and *C. lepidocarpa* clusters.

Allozyme analysis

From the 11 enzyme systems investigated across both taxa, 17 loci were identified and 31 alleles. Six loci (PGI-2, MDH-1, MDH-2, DIA, ME-1 and ME-2) were found to be monomorphic across all taxa thus leaving 11 polymorphic loci (Table 3). Within *C. flava* only two polymorphic loci with a total of four alleles were identified. Of these four

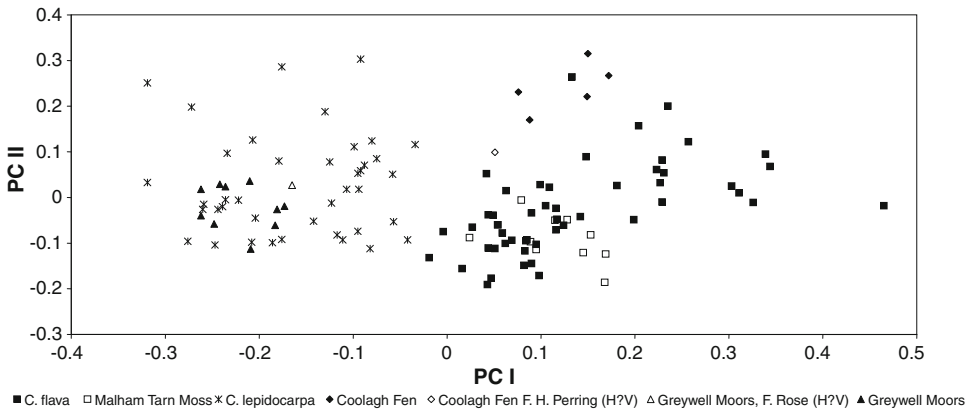

Fig. 1 Scatter plot of PC I versus PC II from PCA of morphometric data of *C. flava*, *C. lepidocarpa* and the putative hybrid populations. *C. lepidocarpa* is separated from *C. flava* along PC I. Malham Tarn individuals cluster with *C. flava*, as do the Coolagh Fen individuals, although these are slightly separated from the mass of *C. flava* specimens. Greywell Moors individuals are located within the *C. lepidocarpa* cluster. All measurements were taken from live material except those denoted (H?V) which were herbarium specimens

Table 3 Mean allele frequencies for *C. flava*, *C. lepidocarpa* and the putative hybrid populations

Allele	Mean allele frequency				
	C. flava	*C. lepidocarpa*	Malham Tarn Moss	Greywell Moors	Coolagh Fen
PGI-1c	0.01	0.12	0.00	0.00	0.00
PGI-1b	0.99	0.86	1.00	1.00	1.00
PGI-1a	0.00	0.02	0.00	0.00	0.00
SKDc	0.93	0.08	1.00	1.00	0.00
SKDb	0.00	0.85	0.00	0.00	0.26
SKDa	0.07	0.08	0.00	0.00	0.71
6PGDc	0.00	0.04	0.00	0.00	0.00
6PGDb	0.00	0.96	0.00	1.00	1.00
6PGDa	1.00	0.00	1.00	0.00	0.00
MDH-1b	1.00	0.91	1.00	1.00	1.00
MDH-1a	0.00	0.09	0.00	0.00	0.00
MNR-2b	1.00	0.73	1.00	1.00	1.00
MNR-2a	0.00	0.27	0.00	0.00	0.00
MNR-1b	1.00	0.91	1.00	1.00	1.00
MNR-1a	0.00	0.09	0.00	0.00	0.00
TPIb	1.00	0.97	1.00	1.00	1.00
TPIa	0.00	0.03	0.00	0.00	0.00
ADHb	1.00	0.15	1.00	1.00	0.00
ADHa	0.00	0.85	0.00	0.00	1.00
PGM-2b	0.00	0.80	0.00	0.00	1.00
PGM-2a	1.00	0.20	1.00	1.00	0.00
PGM-1b	0.00	0.66	0.00	0.00	1.00
PGM-1a	1.00	0.34	1.00	1.00	0.00
GDHb	1.00	0.09	1.00	1.00	0.00
GDHa	0.00	0.91	0.00	0.00	1.00

Note five allozymes fixed in the Malham Tarn Moss population are also fixed or very common in *C. flava* and rare or absent in *C. lepidocarpa*. Greywell Moors has five fixed allozymes which are fixed or very common in *C. flava* and uncommon or absent in *C. lepidocarpa*. Additionally Greywell Moors has one allozyme shared with *C. lepidocarpa* and absent from *C. flava*. Coolagh Fen has five allozymes common to *C. lepidocarpa* and absent or rare in *C. flava*. Coolagh Fen also has one allozyme (*SKDa*) at a high frequency which is only found at low frequency in both *C. flava* and *C. lepidocarpa.* Loci uniform across all taxa are omitted

Table 4 Matrix of mean genetic identity coefficients (Nei 1978) based upon allozyme data and derived from pairwise comparisons of all populations of the *C. flava* agg. sampled

	C. flava	*C. lepidocarpa*	Malham Tarn Moss	Greywell Moors	Coolagh Fen
C. flava	0.9839 (0.9045–1.0000)	0.6610 (0.5854–0.7647)	1.000 (0.9045 –1.0000)	0.8149 (0.7632–0.8235)	0.7593 (0.7550–0.7748)
C. lepidocarpa		0.9329 (0.8406–0.9970)	0.6610 (0.5854–0.7647)	0.7960 (0.7642–0.8371)	0.8218 (0.7759–0.8825)
Malham Tarn Moss				0.8149	0.7593
Greywell Moors					0.8176

Values *in parentheses* are the observed range. *C. flava* and *C. lepidocarpa* have a low genetic identity. Malham Tarn Moss shares an identity with *C. flava*. Greywell Moors has a slightly higher genetic identity to *C. flava* than to *C. lepidocarpa*. Coolagh Fen has a higher genetic similarity to *C. lepidocarpa* than *C. flava*

alleles, one was rare (*PGI-1c*; frequency <0.05). Within *C. lepidocarpa* 23 alleles from ten polymorphic loci were identified. Of these ten, four alleles, *PGI-1a*, *6PGDa*, *6PGDc* and *TPIa*, were rare (frequency <0.05).

Pairwise comparison of populations (Table 4) gave a mean genetic identity for *C. lepidocarpa* of 0.9329 (range 0.8406–0.9970) and 0.9839 (range 0.9045–1.0000) for *C. flava*. Similar pairwise comparisons of the Greywell

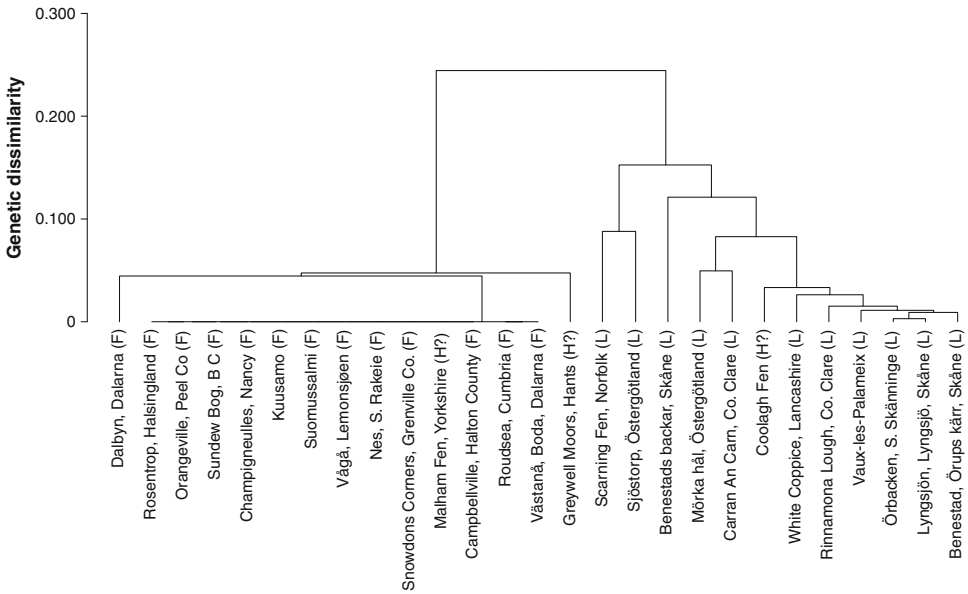

Fig. 2 Phenogram of UPGMA AHC of allele frequencies between sites of *Carex flava* (F), *C. lepidocarpa* (L) and putative hybrids (H?). This shows two major clusters, one containing the *C. lepidocarpa* populations, the other the *C. flava* populations. The Coolagh Fen population falls within the *C. lepidocarpa* cluster. The Malham Tarn population is found in the *C. flava* cluster. Greywell Moors is also in the *C. flava* cluster but is separate from all other populations

Moors, Coolagh Fen and Malham Tarn Moss populations with the *C. lepidocarpa* populations gave mean genetic identities of 0.7960 (range 0.7642–0.8371), 0.8218 (range 0.7759–0.8825) and 0.6610 (range 0.5854–0.7647) respectively. Likewise, pairwise comparisons of the Greywell Moors, Coolagh Fen and Malham Tarn Moss populations with the *C. flava* populations gave mean genetic identities of 0.8149 (range 0.7632–0.8235), 0.7593 (range 0.7550–0.7748) and 0.9901 (range 0.9405–1.000) respectively. For the Malham Tarn Moss population such a pairwise comparison of genetic identity was found to be 1.0000 for all populations with the exceptions of Dalbyn, Västanå (both Swedish populations) and Roudsea Wood (England). For these populations the pairwise genetic identities were 0.9412, 0.9405 and 0.9996 respectively.

A phenogram of UPGMA of Nei's (1978) genetic identity statistics (Fig. 2) clearly shows two distinct clusters that can be ascribed to *C. flava* and *C. lepidocarpa.* The low levels of genetic variation within *C. flava* are reflected by there being no differentiation for the populations ascribed to this taxon with the exception of three sites, Roudsea Wood, Västanå and Dalbyn. By comparison the longer branch lengths in the *C. lepidocarpa* cluster reflect the greater levels of intraspecific variation in this species than seen within *C. flava*.

All of the alleles identified within the Coolagh Fen population are common within *C. lepidocarpa*. Moreover, five alleles (*6PGDb*, *ADHa*, *PGM-1b*, *PGM-2b* and *GDHa*) fixed within this population are totally absent from *C. flava.* The Greywell Moors population contained the allele *6PGDb* common within *C. lepidocarpa* but absent from *C. flava*. However four alleles fixed within the Greywell Moors population (*ADHb*, *PGM-1a*, *PGM-2a*, *GDHb*) are also fixed within *C. flava* and only present at low frequencies (<0.37) within *C. lepidocarpa* (Table 3). Consequently the Greywell Moors population is more closely associated with the *C. flava* cluster although distinct from it (Fig. 2). The Malham Tarn Moss population is fixed for the *6PGDa* allele. This is also fixed in *C. flava* and absent in *C. lepidocarpa*. In addition four other alleles are fixed in both the Malham Tarn Moss population and *C. flava* but at low frequencies in *C. lepidocarpa* (*GDHb*, *ADHb*, *PGM-1a* and *PGM-2a*). Hence the Malham Tarn Moss population is located in the *C. flava* cluster.

Discussion

C. flava and *C. lepidocarpa* differ in both their morphology, as revealed by PCA, and genetically, as revealed by allozymes. Comparable clear separation was found in morphological characters using various methods of multivariate analysis by Crins and Ball (1989a), Stoeva and Štepánková (1990), Pykälä and Toivonen (1994) and Hedrén (2004). Likewise separation between *C. flava* and *C. lepidocarpa* using allozymes partially different from those used in this study was found by Bruederle and Jensen (1991) and Hedrén (2004), all recording a combination of unique alleles and alleles with widely disparate frequencies. The higher levels of allozyme variation in *C. lepidocarpa* compared to *C. flava* recorded here were also found by Bruederle and Jensen (1991), Hedrén and Prentice (1996) and Hedrén (2002, 2004).

Similar genetic identities are found in this study with those obtained from previous work. The intraspecific identity (I) of *C. flava* of 0.98 found here is echoed in the

values of 1.0 and 0.95 recorded by Hedrén (2002) and Bruederle and Jensen (1991) respectively. Within *C. lepidocarpa* Hedrén (2002) recorded a slightly higher value for I (1.0) than that presented here (0.93), although that may reflect the more geographically restricted sample of Hedrén's study. The interspecific genetic identity of *C. flava* and *C. lepidocarpa* of 0.66 in this study is very close to the figure of 0.69 given by Bruederle and Jensen (1991). Both values are higher than the I of 0.42 in Scandinavian material (Hedrén 2002), though this is likely to be the result of Hedrén's exclusion of monomorphic loci. The clear morphological and genetic difference between *C. flava* and *C. lepidocarpa* means that the problematic putative hybrid populations can be fully considered using both sets of evidence.

Morphometric analysis of the Malham Tarn Moss population presented by Blackstock and Ashton (2001) clearly identified this population as being *C. flava* s.str. This was supported by a chromosome count by Davies (1955) giving a haploid number of $n = 30$, typical of *C. flava* s.str., and the high fertility of the plants from this population. A pairwise comparison of genetic identity with the other *C. flava* populations gives a genetic identity of 0.9926 (range 0.9405–1.0000). Indeed, the genetic identity with 13 of the 16 populations studied, including populations from North America, Sweden, France, Finland, Norway, Germany and Belgium, was 1.0000. It is therefore concluded that the Malham Tarn Moss population is *C. flava* s.str.

Allozyme evidence places the Greywell Moors population closer to *C. flava* than *C. lepidocarpa*. There is only one allele that is common to Greywell Moors and *C. lepidocarpa* and absent from *C. flava*. By contrast four alleles fixed within the Greywell Moors population are also fixed within *C. flava* and only present at low frequencies (<0.37) within *C. lepidocarpa*. This is reflected in the mean genetic identity values where that between *C. flava* and the Greywell Moors population (0.8149) is much higher than the mean comparison of genetic identity between *C. flava* and *C. lepidocarpa* (0.6610). These results are in direct contrast with the morphometric data. Specimens collected from Greywell Moors are morphometrically indistinguishable from *C. lepidocarpa*.

Introgressive hybridization between taxa of the *C. flava* agg. growing in close proximity with each other does occur (Schmid 1983). Furthermore *C. lepidocarpa* has a much lower inbreeding coefficient (F_{IS}) than *C. flava* (0.30 and 0.95 respectively; Hedrén 2004). This raises the probability that following hybridization, the resultant F_1 hybrids, albeit with low levels of pollen fertility (Schmid 1980), would be more likely to backcross with the *C. lepidocarpa* present at the site. Hybridization between *C. flava* and *C. lepidocarpa* followed by repeated backcrossing with *C. lepidocarpa* at Greywell Moors would produce individuals with a *C. lepidocarpa* morphology though retaining some *C. flava* allozymes. This signature of introgression would remain in allozyme patterns as these alleles are less likely to be subjected to selective pressures than morphological characteristics and therefore more likely to persist in the population. It is therefore concluded that a population of *C. flava* was once extant at Greywell Moors but subsequently became extinct many years before F. Rose collected his specimen.

The Coolagh Fen population is both morphometrically and enzymatically enigmatic. Within the PCA of morphometric characteristics (Fig. 1) Coolagh Fen clusters along with *C. flava*. This is due to the high loading values associated with the perigynium (BEAKL, PERLE, BRISTLE). However these qualitative characteristics fail to take into account the abrupt tapering of the long, narrow beak into the perigynium body of the Coolagh Fen population as opposed to the more gradual tapering evident in *C. flava.* The longer peduncle to the staminate spikelet and longer, narrower leaves more typical of *C. lepidocarpa*, give some degree of separation of the Coolagh Fen plants from the majority of *C. flava* plants along PC II. Further separation may also be described by the narrower leaf base within this population than is usually found in *C. flava*. A mean plant height of 694 mm and longer perigynium length of 5.12 mm serve to differentiate the Coolagh Fen plants from typical *C. lepidocarpa* (mean plant height of 300–400 mm and perigynium length of 4.5 mm). The overall tall, robust nature of the Coolagh Fen plants may be due to the prevailing ecological conditions of this site which is located on the margins of Lough Corrib within a zonal progression from the lough itself through a dense stand of *Cladium mariscus* (L.) Pohl. Typically *C. lepidocarpa* habitats are characterized by more open vegetative stands with shorter, less dense vegetation such as the M22 *Schoenus nigricans–Juncus subnodulosus* mire (Blackstock and Jermy 2001). Thus morphometrically, the Coolagh Fen population clusters with *C. flava* but some qualitative female reproductive characters are typical of *C. lepidocarpa*.

Enzymatically the Coolagh Fen population is more closely associated with *C. lepidocarpa* than *C. flava* although the genetic identity with the former is still low (0.8218–0.7593 respectively). Four alleles are fixed in *C. flava* but absent in the Coolagh Fen population and a fifth allele (*SKDc*) is present at a high frequency in *C. flava* but absent in Coolagh Fen. In contrast there are no alleles unique to the Coolagh Fen population that are not present in *C. lepidocarpa* although *SKDa* is present at a high frequency (0.7100) in the former but at a very low frequency (0.0791) in the latter. This may reflect an ancestral *C. lepidocarpa* in which selective pressures within this distinctive habitat led to a larger plant than typically found in this taxon. The high frequency of the *SKDa* allele within

the Coolagh Fen population, 0.7100 as compared to 0.080 in *C. flava* and 0.0791 in *C. lepidocarpa*, may reflect the possibility of past hybridization with other members of the *C. flava* agg. Such an allele was found at high frequencies in the closely related *C. demissa* (Blackstock 2007). This species also occurs within the Lough Corrib vicinity.

The findings reached in this study underline the importance of incorporating both morphometric and molecular approaches when trying to identify hybrids within a critical grouping. The Malham Tarn Moss population is identified using both approaches as *C. flava* s.str. However the Greywell Moors population appears to be clearly defined as *C. lepidocarpa* when based upon morphometric data alone. The presence of alleles that are otherwise unique to *C. flava* reveals a more complicated history that strongly suggests that *C. flava* had formerly been extant at this site. This conclusion is further supported by the morphologically intermediate voucher specimen collected by F. Rose over 30 years ago. In contrast the Coolagh Fen population should, if assessed on purely morphological grounds, be assigned to either *C. flava* (based on the PCA plot) or its hybrid (based on utricle morphology). In this case the allozyme data provide no supporting evidence for any historical hybridization events with *C. flava* at this site, and given the lack of any vouchers of *C. flava* from Ireland, lead to the conclusion that the Coolagh Fen population is an unusual form of *C. lepidocarpa* phenotypically adapted to the prevailing environmental conditions, possibly with some introgression from *C. demissa*. The agreement between allozyme and morphometric evidence amongst populations which have been subject to both approaches was considered by Hedrén (2002) to be due to the monophyletic origins of the taxa within the *C. flava* complex. The lack of accord between the two data sets found at Coolagh Fen is therefore unique and perhaps suggests that the taxa can have a polyphyletic origin.

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