The LINNEAN SOCIETY of London BOTANICAL Journal of the Linnean Society

*Botanical Journal of the Linnean Society*, 2014, ••, ••–••. With 1 figure

# Ploidy in the alpine sedge *Kobresia pygmaea* (Cyperaceae) and related species: combined application of chromosome counts, new microsatellite markers and flow cytometry

ELKE SEEBER[1]*, GRIT WINTERFELD[2], ISABELL HENSEN[2,3], TIMOTHY F. SHARBEL[4], WALTER DURKA[5], JIANQUAN LIU[6], YONG-PING YANG[7] and KARSTEN WESCHE[1,3]

[1]*Botany Department, Senckenberg Museum of Natural History Goerlitz, PO Box 300 154, D-02806 Goerlitz, Germany*
[2]*Department of Geobotany and Botanical Garden, Martin-Luther-University Halle-Wittenberg, Am Kirchtor 1, D-06108 Halle (Saale), Germany*
[3]*German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Deutscher Platz 5e, D-04103 Leipzig, Germany*
[4]*Apomixis Research Group, Leibniz Institute of Plant Genetics and Crop Plant Research–IPK, Corrensstraße 3, D-06466 Stadt Seeland, OT Gatersleben, Germany*
[5]*Department of Community Ecology, Helmholtz Centre for Environmental Research–UFZ, Theodor-Lieser-Straße 4, D-06120 Halle (Saale), Germany*
[6]*State Key Laboratory of Grassland Agro-ecosystem, School of Life Sciences, Lanzhou University, Lanzhou, Gansu 730000, China*
[7]*Kunming Institute of Botany, Chinese Academy of Sciences, Heilongtan 650204, Kunming, China*

*Received 26 October 2013; revised 12 May 2014; accepted for publication 29 May 2014*

Polyploidy is a fundamental mechanism in evolution, but is hard to detect in taxa with agmatoploidy or aneuploidy. We tested whether a combination of chromosome counting, microsatellite analyses and flow cytometric measurements represents a suitable approach for the detection of basic chromosome numbers and ploidy in *Kobresia* (Cyperaceae). Chromosome counting resulted in $2n = 64$ for *Kobresia pygmaea* and *K. cercostachys*, $2n = 58$ and 64 for *K. myosuroides*, and $2n = 72$ for *K. simpliciuscula*. We characterized eight microsatellite loci for *K. pygmaea*, which gave a maximum of four alleles per individual. Cross-species amplification was tested in 26 congeneric species and, on average, six of eight loci amplified successfully. Using flow cytometry, we confirmed tetraploidy in *K. pygmaea*. Basic chromosome numbers and ploidy were inferred from chromosome counts and the maximum number of alleles per locus. We consider the basic numbers as $x = 16$ and 18, with irregularities derived from agmatoploidy and aneuploidy. Across all *Kobresia* taxa, ploidy ranged from diploid up to heptaploid. The combination of chromosome counts and microsatellite analyses is an ideal method for the determination of basic chromosome numbers and for inferring ploidy, and flow cytometry is a suitable tool for the identification of deviating cytotypes.  *Botanical Journal of the Linnean Society*, 2014, ••, ••–••.

ADDITIONAL KEYWORDS: 454 sequencing – basic chromosome number – cross-amplification – *Kobresia pygmaea* ecosystem – next-generation sequencing – palaeopolyploidy – Tibetan Plateau.

*Corresponding author. E-mail: elke.seeber@senckenberg.de

## INTRODUCTION

Polyploidy is a major force in plant evolution and occurs frequently across a number of taxa (e.g. Soltis & Soltis, 1999; Soltis, Soltis & Tate, 2004). Wood *et al*. (2009) estimated that 35% of extant vascular plant genera are polyploid, and that 15% of speciation events in angiosperms were facilitated by a duplication of the chromosome set. The multiplication of entire genomes is usually regarded as being associated with higher tolerance to environmental stress (e.g. Grant, 1981; Goldman *et al*., 2004), because polyploid taxa are known to be relatively more abundant than diploids in arctic and alpine environments (Stebbins, 1985; Hijmans *et al*., 2007). Polyploidization therefore plays a pivotal role in the diversification of plants, especially in mountain habitats (Burnier *et al*., 2009).

Several genera of the family Cyperaceae are considered to be polyploids (Roalson, 2008), although ploidy is hard to verify in this group because species have variable chromosome numbers (Hipp, Rothrock & Roalson, 2009b, and references therein) as a result of agmatoploidy (the fission and fusion of chromosomes, Davies, 1956; Luceño & Castroviejo, 1991) and aneuploidy (the decrease or increase by single chromosomes, Wolfe, 2001). Members of the family Cyperaceae are of ecological importance as they are major components of many ecosystems (Leck & Schütz, 2005), especially in arctic and alpine habitats, arousing a new interest in the biology of the group. Sedges show large species diversity and some are also economically important (especially species of *Cyperus* L. and related taxa).

In the present study, we investigated the basic chromosome number and ploidy in the sedge genus *Kobresia* Willdenow. This genus comprises *c*. 60 species (Zhang, 2001), which are widespread and locally dominant in the Northern Hemisphere, with the highest species diversity occurring in alpine regions of the Himalayas and adjacent mountain ranges (Dahlgren, Clifford & Yeo, 1985; Zhang, Liang & Dai, 1995). Few cytological studies are available, mainly because of the limited access to growing tissue. Chromosome counts are rarely reported and vary from $2n = 32$ in *K. schoenoides* Boeckeler to $2n = c.$ 122 in *K. nepalensis* (Nees) Kük. (Poghosian, Narinian & Voskanian, 1971; Hoshino, Rajbhandari & Ohba, 2000). Basic chromosome numbers of the genus remain under debate, with estimates ranging from dibasic with $x = 26$ and 36, to tribasic with values of $x = 8$, 9 and 13 (Darlington & Wylie, 1955; Mehra & Sachdeva, 1976). No study has yet specifically examined the origin of ploidy in *Kobresia*, but studies in the closely related *Carex* L. revealed that autopolyploidy rather than allopolyploidy dominates (Heilborn, 1939). This is also regarded as the predominant mechanism across Cyperaceae as a consequence of the peculiar pollen formation in the family (Heilborn, 1939). One exception based on chromosome counts is a possible allopolyploid origin for *K. nepalensis* from ancestors such as *K. myosuroides* (Vill.) Fiori and *K. esenbeckii* (Kunth) Noltie (Yano *et al*., 2011).

Three methods are frequently used to determine ploidy: chromosome counting, microsatellite analyses and flow cytometric measurements. The traditional approach, using direct analysis of metaphase chromosomes, provides the actual chromosome number. However, in Cyperaceae, interpretations of counts are difficult because of agmatoploidy and aneuploidy. A fundamental constraint is the availability of growing tissue, especially in rare species or those growing under conditions that are not easily reproduced.

Second, nuclear microsatellite markers (simple sequence repeats, SSRs) can successfully be used in ecological studies in diploid and polyploid species (Pfeiffer *et al*., 2011; Ritz & Wissemann, 2011; Avolio *et al*., 2012). Their co-dominant expression enables the detection of heterozygotes and allows for the linking of the maximum number of alleles found in one individual to ploidy (Besnard *et al*., 2008; Stark *et al*., 2011) and, where the chromosome number of the species is known, to the basic chromosome number. This method requires only a few milligrams of dried plant material, but the statistical probability of complete heterozygotes decreases with increasing ploidy, and there are numerous genera in which suitable SSR loci are not yet available. The use of microsatellite markers developed for other taxa is potentially possible, but cross-amplification rates are typically low, even between congeneric species, and they are greatly reduced in monocots relative to eudicots (Peakall *et al*., 1998; Barbará *et al*., 2007). In addition, the probability of null alleles increases as a result of mutation accumulation in the primer binding sequences, and subsequent partial heterozygotes introduce ambiguity (Erler, Stoneking & Kayser, 2004). Therefore, it is critical to ensure reliable amplification in interspecific tests when microsatellites are to be used to assess ploidy. In any case, such methodological constraints can be minimized by using a sufficient number of samples and reliable loci.

Third, the ploidy of species can be determined from the DNA contents. Doubling of the chromosome numbers usually leads to an increase or doubling of the DNA contents. Recently, such changes have become easy to determine using flow cytometry, in which only small amounts of fresh/dried tissue or seeds suffice (Suda & Trávníček, 2006). However, DNA content appraisal using flow cytometry does not reveal ploidy directly, and reference samples with known ploidy are required (e.g. Pellicer *et al*., 2012).

In this study, we chose *K. pygmaea* C.B.Clarke ex Hook.f. for the initial establishment of microsatellite protocols. This species is the most ecologically and economically important representative of the genus, covering more than 450 000 km$^2$ of the Tibetan Plateau and adjacent areas (Miehe *et al*., 2008, 2011). It is the dominant species in the second largest alpine ecosystem and forms the basis for Tibetan livelihoods and husbandry. We tested the reliability of the selected primers in the other species. The combination of the three methods was then used to assess: (1) the ploidy of *K. pygmaea*; (2) the basic chromosome numbers in *Kobresia*; and (3) the frequency of polyploidy in *Kobresia*.

## MATERIAL AND METHODS

### Plant material

In summer 2009, we collected plant material of *K. pygmaea* near Kema village (31.27418°N, 92.11037°E, 4484 m a.s.l.), 20 km south of Nagqu, Tibetan Autonomous Region. The site represents the core of the species distribution in which polymorphism is assumed to be high and relatively unaffected by genetic drift. *Kobresia pygmaea* dominates the vegetation with up to 80% cover. Seeds were collected over an area of 100 × 100 m$^2$, stored at room temperature and mechanically scarified to break physical dormancy. Incubation (12 h light at 20 °C, 12 h darkness at 5 °C) yielded ten individuals, from which leaf material was sampled. Only one plant recovered after harvesting of leaf material, and was hence kept for chromosome counting. Another 100 tissue samples (3–5 mg) were collected *in situ* at a 100-m$^2$ plot and dried.

We retrieved samples of 24 *Kobresia* spp., plus two subspecies from specimens that had been stored for no longer than 20 years, from the High Asia Project Herbarium Marburg (Supporting Information, Table S1). We obtained living *K. cercostachys* C.B.Clarke, *K. simpliciuscula* Mack. and *K. myosuroides* from the Alpine Botanical Garden 'Brockengarten' (National Park Harz, Germany) and kept them at the Botanical Garden Halle. Voucher specimens are lodged at the Herbarium Senckenbergianum Görlitz GLM. Together, the 27 species represent 45% of all described *Kobresia* spp.

The nomenclature of *Kobresia* is still not settled. Here (Supporting Information, Tables S1 and S4; Table 2) we follow the current *Flora of China* (Zhang & Noltie, 2010), various publications on the Cyperaceae of the Canadian Arctic Archipelago (Aiken *et al*., 2007a, b) and a flora of Germany (Jäger & Werner, 2002).

### Chromosome numbers

Chromosome counts were conducted in one individual each of *K. pygmaea*, *K. cercostachys* and *K. simpliciuscula* and two individuals of *K. myosuroides*. Excised root tips were treated in iced water for 20 h to accumulate metaphases, and then fixed in absolute ethanol–glacial acetic acid (3 : 1) for 3 h and stored in absolute ethanol at –20 °C until preparation. Enzyme-treated root tips (Schwarzacher, Ambros & Schweizer, 1980) were squeezed on slides in a drop of 45% propionic acid with 2% carmine according to Winterfeld & Röser (2007). Photographs of metaphase chromosomes were taken on a Zeiss Axiophot microscope using a Zeiss Axiocam HRC CCD camera employing Zeiss Axiovision software. Additional information on chromosome numbers was collated from the literature.

### Identification of microsatellite loci

Total genomic DNA was extracted from a pooled sample of ten seed-grown *K. pygmaea* plants following the 2 × cetyl trimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1987). DNA quality was verified by electrophoresis on agarose gel and by spectrophotometry (NanoDrop, Thermo Fisher Scientific, Wilmington, NC, USA).

The sample was sent to the pyrosequencing facilities of the Duke Institute for Genome Sciences and Policy (Durham, NC, USA) for further preparation according to Margulies *et al*. (2005). The shotgun library was prepared from random genomic DNA fragments using the Titanium system (Roche Diagnostics, Pleasanton, CA, USA), tagged with multiplex identifiers and multiplexed (equivalent to one-eighth of the library pool) to run on a PicoTiterPlate of a Roche 454 Genome Sequencer FLX Instrument.

We screened the sequences for di-, tri-, tetra-, penta- and hexanucleotide motifs with at least ten, seven, six, five and four repeats with the software MSATCOMMANDER-1.03 for Mac OS X (Faircloth, 2008). Primers were defined in the flanking regions of the microsatellites using Primer3 (Rozen & Skaletsky, 1999), implemented in MSATCOMMANDER. The forward primer was tagged with either a CAG-tag (5′-CAGTCGGGCGTCATCA-3′) or an M13(-21) tag (5′-TGTAAAACGACGGCCAGT-3′), according to Schuelke (2000). Where possible, a GTTT tail was attached to the 5′ end of the reverse primer to promote adenylation. We selected those primers that yielded products between 100 and 410 bp for use with the MegaBace ET400-R size standard (Amersham Bioscience, Amersham, UK). Primers with redundancy of sequences were manually omitted. We chose 20 of the remaining primer pairs, representing different repeat motifs and product sizes, checked

that they were designed from high-quality reads and ordered them from Metabion (Martinsried, Germany). These were screened with a subset of five individuals on a MegaBace 1000 automatic sequencer (Amersham Bioscience) following the procedure described by Schuelke (2000). Eight loci showed scorable polymorphic patterns, and were ordered without the tag sequence with either HEX or FAM fluorescent labels attached to the forward primer (Metabion) for further use.

### Characterization of microsatellite loci

DNA extraction followed a modified 2 × CTAB protocol with a modified extraction buffer [2% alkyl trimethyl ammonium bromide, 0.1 M Tris-HCl, 0.02 M disodium-ethylene diamine tetraacetate (EDTA), pH 8.0, 1.4 M NaCl, 1% polyvinylpyrrolidone (PVP)]. We used 1–3 mg of dried leaves, and the precipitation period was extended to 24 h at –20 °C. To digest RNA, DNA pellets were dissolved in 20 µl 1 × TE buffer with RNase (10 µg $ml^{-1}$) and incubated at 37 °C for 30 min.

Polymerase chain reaction (PCR) was performed in a total volume of 25 µl containing 10 ng of DNA, 1 U Taq polymerase (DreamTaq™ DNA Polymerase, Fermentas, St. Leon-Rot, Germany), 3 µl of 10 × reaction buffer (Fermentas), 3 µl magnesium chloride (25 mM), 5 mM of each deoxynucleoside triphosphate (dNTP) and 2.5 pmol of each of the forward and reverse primers. We ran the following PCR programme on a Mastercycler epgradient thermocycler (Eppendorf, Hamburg, Germany): denaturation at 94 °C for 4 min, followed by two cycles at 94 °C (45 s), 60 °C (45 s) and 72 °C (45 s), 18 touchdown cycles at 94 °C (45 s), 59 °C (45 s; decreasing by 0.5 °C per cycle), 72 °C (45 s), 20 cycles at 94 °C (30 s), 50 °C (30 s) and 72 °C (45 s), and a final extension at 72 °C for 5 min (Collada *et al*., 2004). Products from singleplex PCRs were pooled for simultaneous runs of four loci on a MegaBace 1000 (Amersham Bioscience) using the MegaBace ET400-R size standard (Amersham Bioscience). We scored the amplification products with the Fragment Profiler version 1.2 (Amersham Bioscience) and revised manually.

We calculated individual ploidy for the 100 *in situ K. pygmaea* samples using the R package POLYSAT (Clark & Jasieniuk, 2011). The maximum number of alleles was four, suggesting tetraploidy. However, 17% of the samples had fewer than four alleles across all eight loci, and so ploidy could not be unequivocally assigned. POLYSAT further indicated that 38 of the remaining samples had identical genetic phenotypes, and thus carried no additional information. These samples were therefore omitted from the study. To define the exact genotype of partial heterozygotes, we applied the MAC-PR method (Esselink, Nybom & Vosman, 2004), which indicated ambiguous results for one or more loci in 20 cases, leaving 25 samples for further analysis.

We calculated the number of alleles per locus and heterozygosity using the Bayesian approach of the program AUTOTET (Thrall & Young, 2000), which accounts for polysomic inheritance and double reduction of autopolyploids, as opposed to disomic inheritance in allopolyploids. As a result of a lack of precedence of studies on segregation mode, we calculated the expected heterozygosity and fixation index based on chromosome segregation and chromatid segregation, assuming maximum double reduction ($\alpha = 0.143$). Deviations from Hardy–Weinberg equilibrium (HWE) were tested with chi-squared goodness-of-fit tests for observed-to-expected genotype frequencies implemented in AUTOTET.

With the same PCR conditions, we also tested the transferability of the primers to 26 *Kobresia* spp. (Supporting Information, Table S1) from the Himalayas and Europe. We made a rough estimate of ploidy with POLYSAT for those *Kobresia* spp. that had amplification products for at least four loci. Estimates were made with respect to chromosome numbers where available. For the four species we had grown, ploidy estimates were based on our chromosome counts.

### Flow cytometric measurements

The DNA content of the 25 *K. pygmaea* samples included in the AUTOTET analysis and the voucher specimens was examined using flow cytometry following Otto (1990) with the following modifications: *c*. 1 mg of the silica-dried tissue was ground individually together with 50 µl of Otto I isolation buffer (0.1 M citric acid monohydrate, 0.5% Tween 20 dissolved in $H_2O$, pH 2.5) for 2 min at 100 strokes per minute using a Geno-Grinder 2000 (SPEX CertiPrep). Another 250 µl of isolation buffer was added, followed by a filtration step (Partec 30-µm mesh-width filters). We mixed 80 µl of the filtrate with 80 µl of staining buffer, Otto II [0.4 M $Na_2HPO_4.12H_2O$, 4 µg $ml^{-1}$ 4′,6-diamidino-2-phenyl-indole (DAPI), pH 8.5], and incubated the mixture for 10 min on ice before analysis on a Partec PAII flow cytometer (Partec GmbH, Münster, Germany). We determined the single DNA contents in relation to an external standard (set at a peak position of 108.3 at a gain of 535) from silica-dried tissue of a *K. pygmaea* specimen of known ploidy (collection number 161962, Supporting Information, Table S1) according to Doležel, Greilhuber & Suda (2007). An external standard was used because we had no internal standard available meeting the minimum requirements of: (1) leaf material being available in sufficient amounts; (2) working well under the

protocol optimized for *K. pygmaea*; and (3) not having an overlap in peak position. Results were, however, verified by a second round of measurements against an internal standard which became available at a later stage of the study.

## RESULTS

### Chromosome numbers

Chromosome counting in *K. pygmaea* and *K. cercostachys* resulted in $2n = 64$. For *K. myosuroides* and *K. simpliciuscula*, we confirmed earlier counts. *Kobresia myosuroides* had variable chromosome numbers with $2n = 58$ and 64 (Fig. 1), whereas *K. simpliciuscula* showed $2n = 72$.

### Microsatellite analyses

Sequencing of *K. pygmaea* resulted in 140 972 reads at a medium length of 444 bases, totalling 62.5 Mb of DNA. Scoring for microsatellites yielded 1643 unique loci. As a result of the limitations in primer design, such as the product size or self-complementarity, tagged primers could be generated for 181 motifs only. We pre-screened 20 primer combinations, stretching over the whole size range and representing different

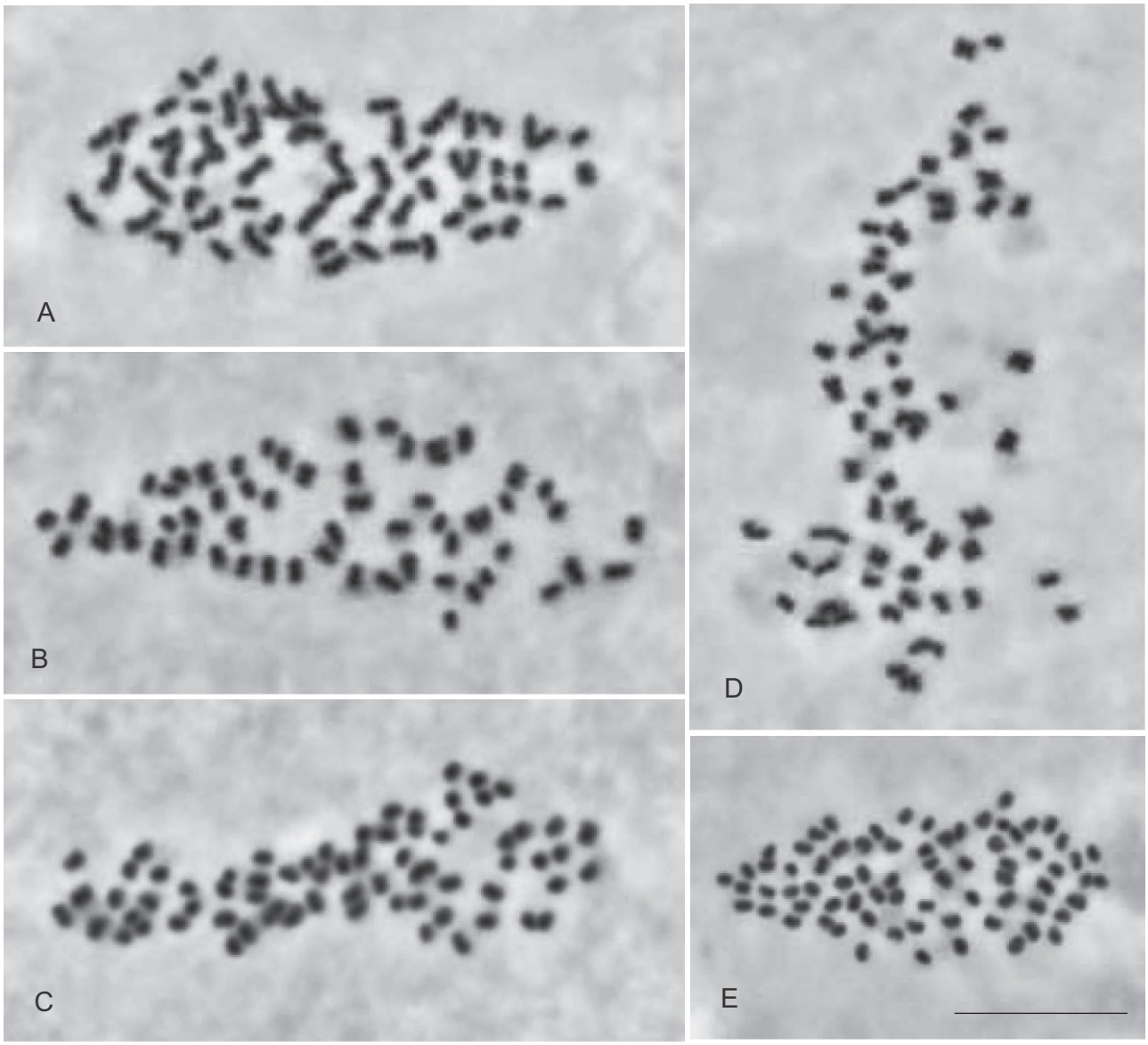


**Figure 1.** Mitotic metaphase chromosomes of *Kobresia pygmaea* (A, $2n = 64$), *K. myosuroides* (B, $2n = 58$), *K. myosuroides* (C, $2n = 64$), *K. cercostachys* (D, $2n = 64$) and *K. simpliciuscula* (E, $2n = 72$). Scale bar, 5 μm.

repeat motifs. Eight primer pairs (Table 1) gave interpretable polymorphic band patterns. Information on markers yielding questionable results (12, Table S2) and those not yet tested (161, Table S3) is listed in Supporting Information. Corresponding DNA sequences are available at the DNA Data Bank of Japan (DDBJ). Further details and additional sequence data are freely available on request to the corresponding author.

Across the eight loci and 25 *K. pygmaea* samples, the number of alleles per locus ranged from nine to 15, with an average of 12 alleles (Table 1). Observed heterozygosity varied from 0.19 to 0.81, with a mean of 0.62. Under the assumption of chromosomal segregation, the expected heterozygosity [$H_E(C_e)$ = 0.77] was, on average, slightly higher than that predicted in the chromatid segregation mode [$H_E(C_d)$ = 0.72]. In terms of chromosome segregation, the chi-squared goodness-of-fit test indicated significant departures from HWE at six loci, whereas chromatid segregation resulted in deviations at four loci. The maximum number of different alleles per locus in any given *K. pygmaea* individual was four.

In total, primers generated products in 74% of the 224 tested cross-amplifications (Supporting Information, Table S4). On average, each *Kobresia* sp. produced reliable results at six of eight loci. All eight loci could be amplified in *K. nepalensis*, *K. setschwanensis* Hand.-Mazz. and *K. williamsii* T.Koyama, whereas *K. macrantha* Boeckeler yielded only two successful cross-amplifications. Transferability of loci ranged from being successful in only nine species at Kp_8 to 27 species at Kp_9. Taking only the successful cross-amplifications into account, the average number of alleles amplified per locus and individual ranged from 1.2 (± 0.4 SD) for Kp_7 to 1.8 (± 1.0 SD) for Kp_9, and the proportion of homozygotes was *c*. 39%. Allele distributions imply that nine species (37.5%) are at least tri- or tetraploid.

### Flow cytometric measurements

The peak position of the 25 analysed *K. pygmaea* samples was at 114.0 (± 10.2 SD). DNA contents were regarded as equal to that of the external standard with a known chromosome number (108.3; Supporting Information, Fig. S1). Our attempt to analyse the voucher specimens yielded reliable peaks only in *K. pygmaea* (65.8), *K. schoenoides* (73.9) and *K. robusta* Maxim. (70.2).

### Basic chromosome numbers and ploidy

Given that a maximum of four alleles per locus was found in 100 samples, we consider *K. pygmaea* as tetraploid to conform to $2n = 4x = 64$, resulting in a basic chromosome number of $x = 16$. The chromosome numbers of the individuals of *K. cercostachys* ($2n = 64$) and *K. myosuroides* ($2n = 58, 64$) are similar or of the same order of magnitude as *K. pygmaea*. These two species are therefore also predicted to be tetraploid, although samples produced a maximum of three alleles at seven loci and two alleles at six loci, respectively.

The chromosome number of *K. simpliciuscula* ($2n = 72$) does not represent a multiple of $x = 16$. Considering a deviation of eight chromosomes to either $2n = 4x = 72$ or $2n = 5x = 72$, and acknowledging that the genus is believed to be polybasic, we suggest $x = 18$ as a second basic number. Hence, *K. simpliciuscula* is probably tetraploid with $2n = 4x = 72$. Pentaploidy to heptaploidy was indicated for *K. duthiei* C.B.Clarke ex Hook.f and *K. nepalensis*. The combination of basic numbers and allele distributions (Table 2) increased the rate of polyploids by another 18.1%, yielding 55.6% in total.

## DISCUSSION

### Chromosome numbers

We contribute the first available chromosome numbers for *K. pygmaea* and *K. cercostachys* and confirm former counts in *K. myosuroides* and *K. simpliciuscula*. Our data are in the reported range of $2n = 32$ in *K. schoenoides* and $2n = c.$ 122 in *K. nepalensis* (Poghosian *et al*., 1971; Hoshino *et al*., 2000).

In our study, *K. myosuroides* showed a difference of about six chromosomes in two individuals considered to have the same ploidy (Table 2). Similar patterns were observed in *K. sibirica* Turcz. ex Boeckeler, *K. simpliciuscula* and *K. nepalensis*, representing all species with two or more published chromosome counts. Pluralities of cytotypes are also known from > 100 *Carex* spp., with a difference of up to ten haploid chromosomes from the highest to the lowest count in different individuals (Luceño & Castroviejo, 1991; Hipp *et al*., 2009b, and references therein). Small variations even occur within the same individual (Luceño, 1994). Three highly unusual features of Cyperaceae among angiosperms allow for this: diffuse kinetochores (Heilborn, 1924); post-reductional meiosis (Wahl, 1940); and the production of a single pollen grain (pseudomonad) per pollen mother cell (Hipp *et al*., 2009b). Two possible mechanisms (agmatoploidy and aneuploidy) explain chromosome number changes, and both increasing and decreasing chromosome numbers can occur during speciation (Hipp *et al*., 2009b). The potential for a rapid chromosomal evolution was suggested as the main driving factor of speciation in *Carex* (Roalson, Columbus & Friar, 2001; Hipp *et al*., 2007), and

**Table 1.** Characteristics of microsatellite markers, observed allele numbers and heterozygosity in tetraploid *Kobresia pygmaea*. The sequence of the forward primer represents a fusion of a universal tag sequence and the selective primer sequence. The tag sequence is highlighted in bold; the expected size is given including the tag. *A*, number of alleles; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity; *F*, fixation indices considering $C_e$ (chromosome segregation) and $C_d$ (chromatid segregation); significance for deviations from Hardy–Weinberg equilibrium ($\chi^2$ goodness-of-fit test): $^*P < 0.05$

| Locus | GenBank accession no. | Primer sequence (5′–3′) | Label | Repeat motif | Expected size (bp) | *A* | $H_O$ | Chromosome ($C_e$) | | Chromatid ($C_d$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | $H_E(C_e)$ | $F(C_e)$ | $H_E(C_d)$ | $F(C_d)$ |
| Kp_7 | AB827630 | F: **TGTAAAACGACGGCCAG**TTTCCCTCCATAGAAATCAC<br>R: GTTTCTTGAGAGAGGGAGAATCGTTTC | FAM | $(AAG)_7$ | 300 | 10 | 0.49 | 0.55 | 0.10 | 0.51 | 0.04 |
| Kp_8 | AB827631 | F: **TGTAAAACGACGGCC**AGTGTAGGAAAGCGAAGAAG<br>R: TCACAGAGTCACAGGGCAGT | FAM | $(GA)_{14}$ | 345 | 15 | 0.54 | 0.85* | 0.37 | 0.80* | 0.32 |
| Kp_9 | AB827632 | F: **TGTAAAACGACGGCCA**GTACAACCCAAATCTGAACC<br>R: GTTTCTTCCAATGGAGAAAGTCTTGTC | FAM | $(ACT)_7$ | 403 | 13 | 0.69 | 0.78* | 0.12 | 0.72* | 0.05 |
| Kp_11 | AB827633 | F: **TGTAAAACGACGGCCAG**TGTCATTTCTCCCTGATTTC<br>R: AGGACTTTGACCCTATGATC | FAM | $(AAT)_9$ | 405 | 14 | 0.80 | 0.80 | 0.00 | 0.75 | –0.07 |
| Kp_16 | AB827634 | F: **CAGTCGGGCGTCATC**ATTAACGGCTCAGTCATGTG<br>R: GCCCAAACATAAATAAGGTG | HEX | $(AT)_{10}$ | 140 | 11 | 0.65 | 0.82* | 0.20 | 0.76 | 0.14 |
| Kp_18 | AB827635 | F: **CAGTCGGGCGTCA**TCAAGTTGAAGGGTGTCATAC<br>R: AACATCAGCAACAGCAGCAG | HEX | $(ATT)_{20}$ | 178 | 9 | 0.19 | 0.73* | 0.74 | 0.68* | 0.73 |
| Kp_19 | AB827636 | F: **CAGTCGGGCGTCATCA**GCTACCCACAGATTAAGTAGC<br>R: AACAGATAGACCCGCCATGT | HEX | $(AG)_{15}$ | 203 | 14 | 0.78 | 0.82* | 0.05 | 0.77 | –0.02 |
| Kp_21 | AB827637 | F: **CAGTCGGGCGTCATCA**CCAGGCATACATTTGGAC<br>R: ACTGCTTGGTTGTTGAGCC | HEX | $(CT)_{12}$ | 173 | 10 | 0.81 | 0.83* | 0.02 | 0.77* | –0.05 |
| | | Mean | | | | 12.00 | 0.62 | 0.77 | 0.20 | 0.72 | 0.14 |
| | | SD | | | | 2.27 | 0.21 | 0.10 | 0.19 | 0.09 | 0.19 |

**Table 2.** Diploid ($2n$) and haploid ($n$) chromosome numbers, expected basic chromosome number $x$, maximum number of alleles in cross-amplification $n_A$ (from Supporting Information, Table S4) and potential ploidy level $p$ in species of *Kobresia*. Data from the four species, in which chromosome counts and microsatellite analyses were retrieved from the same individual, are highlighted in bold. Estimation of ploidy is based on chromosome numbers and maximum number of alleles in cross-amplification. Question marks indicate ambiguous cases

| Species | $2n$ ($n$) | Reference | $x$ | Maximum $n_A$ | $p$ |
|---|---|---|---|---|---|
| ***Kobresia pygmaea* C.B.Clarke ex Hook.f.** | **64** | **Present study** | ***16*** | **4** | **4** |
| *Kobresia capillifolia* C.B.Clarke | | | | 4 | ≥ 4 |
| ***Kobresia cercostachys* C.B.Clarke** | **64** | **Present study** | **16** | **3** | **4** |
| *Kobresia cuneata* Kük. | | | | 2 | ≥ 2 |
| *Kobresia curvata* C.B.Clarke | 50 | Yano *et al*. (2011) | 16? | | 3 |
| | | | 26? | | 2 |
| *Kobresia duthiei* C.B.Clarke ex Hook.f. | *c*. 84 | Yano *et al*. (2011) | 16? | 2 | 5 |
| | | | 26? | | 3 |
| *Kobresia esenbeckii* (Kunth) Noltie | 66 | Yano *et al*. (2011) | 16? | 3 | 4 |
| *Kobresia filifolia* C.B.Clarke | *c*. 60 | Probatova & Sokolovskaya (1988) | 16? | n.c. | 4 |
| *Kobresia fragilis* C.B.Clarke | | | | 2 | ≥ 2 |
| *Kobresia humilis* (C.A.Mey. ex Trautv.) Serg. | | | | n.c. | |
| *Kobresia kansuensis* Kük. | | | | 2 | ≥ 2 |
| *Kobresia karakorumensis* Dickoré | | | | 1 | ≥ 2 |
| *Kobresia macrantha* Boeckeler | | | | n.c. | |
| ***Kobresia myosuroides* (Vill.) Fiori** | **58, 64** | **Present study** | **16** | **2** | **4** |
| | 52–59 | Heilborn (1939) | 18? | | 2,3 |
| | *c*. 36, 52, 56, 58 (29), 60–66 | Reviewed in Aiken *et al*. (2007b), Elven (2007 and onwards) and Roalson (2008) | 26? | | 2 |
| | | | 29? | | 2 |
| | (26) | Tischler (1950) | 26 | | 2 |
| *Kobresia nepalensis* (Nees) Kük. | *c*. 122 | Hoshino *et al*. (2000) | 19? | 3 | 6 |
| | *c*. 114 | Yano *et al*. (2011) | 18? | | 6 |
| | | | 16? | | 7 |
| *Kobresia prainii* Kük. | | | | 2 | ≥ 2 |
| *Kobresia pusilla* Ivanova | | | | 2 | ≥ 2 |
| *Kobresia robusta* Maxim. | | | | 2 | ≥ 2 |
| *Kobresia royleana* Nees ex Boeckeler | (40) | Mehra & Sachdeva (1976) | 16 | 3 | 5 |
| *Kobresia schoenoides* Boeckeler | 32 | Poghosian *et al*. (1971) | 16 | 2 | 2 |
| *Kobresia setschwanensis* Hand.-Mazz. | | | | 4 | ≥ 4 |
| *Kobresia sibirica* (Turcz. ex Ledeb.) Boeckeler | *c*. 54, 58, 62 | Reviewed in Roalson (2008) | 16? | | 4 |
| | | | 18? | | 3 |
| ***Kobresia simpliciuscula* (Wahlenb.) Mack.** | **72** | **Present study** | **18** | **2** | **4** |
| | 76 (38) | Knaben & Engelskjøn (1967) | 19? | | 4 |
| | 58, 60, 64, 70–75 (*c*. 37) | Reviewed in Elven (2007 and onwards) and Roalson (2008) | 16? | | 4 |
| *Kobresia stolonifera* Y.C.Tang ex P.C.Li | | | | 3 | ≥ 3 |
| *Kobresia tibetica* Maxim. | | | | 2 | ≥ 2 |
| *Kobresia uncinioides* (Boott) C.B.Clarke | | | | 3 | ≥ 3 |
| *Kobresia vidua* (Boott ex C.B.Clarke) Kük. | | | | 2 | ≥ 2 |
| *Kobresia williamsii* T.Koyama | | | | 2 | ≥ 2 |
| *Kobresia yadongensis* Y.C.Yang | | | | 2 | ≥ 2 |

n.c., not calculated due to insufficient cross-amplification success.

possibly in all Cyperaceae. Nonetheless, the basic chromosome number within a species is considered to be unaffected, but smaller changes in chromosome numbers cannot be directly attributed to polyploidy because of such features.

### Microsatellite analyses

Here, we present the first microsatellite loci available in *Kobresia*. In *K. pygmaea*, the allelic diversity of the microsatellites was high for most loci (mean $A = 12.00$). This is a typical pattern in autopolyploid species in which high heterozygosity and allelic diversity result from polysomic inheritance (Soltis & Rieseberg, 1986; Soltis & Soltis, 1995; Gauthier, Lumaret & Bédécarrats, 1998). Multiple origins from the same diploid progenitor species have been reported for bryophytes (Wyatt *et al*., 1988), ferns (Ranker *et al*., 1989) and angiosperms (Brochmann, Soltis & Soltis, 1992; Parisod & Besnard, 2007; Wu *et al*., 2010), which have continuously increased intra-autopolyploid diversity by introducing new maternal lineages (Soltis & Soltis, 1999). Therefore, even if we rejected an allopolyploid origin for *K. pygmaea* following Heilborn (1939), multiple origins from the same diploid ancestor or introgression from other species potentially played a role in the diversification history of *K. pygmaea*, and possibly in the entire genus (Waterway, 1994; Zhang, 2001; Yano *et al*., 2011).

Similarly, Zhao *et al*. (2006) reported high levels of within-population genetic diversity for five *Kobresia* spp. from the eastern regions of the Qinghai–Tibet Plateau [*K. setchwanensis*, *K. humilis* (C.A.Mey. ex Trautv.) Serg., *K. kansuensis* Kük., *K. royleana* Nees ex Boeckeler, *K. tibetica* Maxim.; in increasing order of diversity]. We assume *K. kansuenis* and *K. tibetica* to be diploid, whereas *K. royleana* and *K. setchwanensis* are tri- or tetraploid (Table 2). Thus, high genetic diversity seems to be a general feature of *Kobresia*, regardless of ploidy, which was formerly interpreted as an indication of frequent input of genets from sexual reproduction.

The observed heterozygosity of *K. pygmaea* is variable, ranging from 0.19 to 0.81 for a given locus. The comparison of the observed-to-expected genotype frequencies, independent of the supposed segregation mode, indicated deviations from HWE. The existence of null alleles could be one explanation. The analyses of microsatellite loci can, however, also be affected by the loss of the associated chromosomes (aneuploidy), or breakage within the amplified locus resulting in a breakdown. Such minor changes in chromosome numbers or structure cannot be detected by flow cytometric measurements, but by patterns formed by chromosomes in meiotic metaphase I (Hoshino & Okamura, 1994). However, the impact of these complex cytological features of Cyperaceae on measures of genetic diversity decreases with increasing number of included loci. To the best of our knowledge, they have never been considered as having a significant impact in molecular studies. Some caution may still be needed, because the results presented here were obtained from a limited number of samples from one population, and this may vary between populations (Liu *et al*., 2009).

Cross-amplification success was high (74%) for most of the loci in the *Kobresia* spp. analysed, suggesting that microsatellites can be transferred across the whole genus. Studies in *Schoenoplectus* (Rchb.) Palla, *Carex* and *Scirpus* L. obtained even higher rates at 81%, 91% and 100%, respectively (Blum *et al*., 2005; Zhou *et al*., 2009; Hipp *et al*., 2009a), suggesting that cross-species transferability is generally high in Cyperaceae, in contrast with other families (Peakall *et al*., 1998; Barbará *et al*., 2007).

In the present study, proportions of polymorphic loci in *Kobresia* spp. were twice as high as in other monocots reviewed in Barbará *et al*. (2007). Nevertheless, assumptions on ploidy solely based on allele number would have underestimated ploidy in most cases.

### Flow cytometric measurements

All 25 tested *K. pygmaea* samples belong to the tetraploid cytotype, but it remains unknown as to whether the 17 samples with fewer than four alleles at the eight loci represent partial heterozygotes or belong to a different cytotype. The presence of multiple cytotypes is common in alpine species (Gauthier *et al*., 1998; Baack, 2004; Schönswetter *et al*., 2007) and has also been described for the Tibetan Plateau (Yuan, Küpfer & Zeltner, 1998; Yamane, Yasui & Ohnishi, 2003; Cui *et al*., 2008; Meng *et al*., 2010) with niche differentiation being a frequent pattern (for example, Wu *et al*., 2010, and references therein). However, Zhang (2001) suggested *K. simpliciuscula* and *K. myosuroides* as possible evolutionary progenitors of *K. pygmaea*, based on morphological parameters. These two species are already tetraploid (Table 2). Further broad-scale attempts and studies on chromosome segregation are needed to specify whether other cytotypes of *K. pygmaea* exist and how tetraploidy evolved.

The analysis of voucher specimens according to the standard protocol was unsuccessful in most cases. Among other factors, ploidy itself can influence the success of analyses because polyploid species have a shorter flow cytometric lifetime as the yield of intact nuclei can decrease more quickly (Suda & Trávníček, 2006). In this study, we used vouchers of variable age and revealed different ploidy in *Kobresia* spp. It

would have been difficult to identify artefacts or their sources, and we thus made no efforts to customize the standard protocol for the other species.

In any case, considering that all samples worked in the SSR analyses, flow cytometry seems to be more vulnerable to the age and storage conditions of specimens relative to molecular analyses. Thus, in our study, flow cytometry allowed for the confirmation of tetraploidy for relatively fresh *K. pygmaea* samples stored for the same time under comparable conditions, but provided no additional information on ploidy for *Kobresia* spp.

### Basic chromosome numbers and ploidy

Based on formerly published basic chromosome numbers, ranging from $x = 8$ to $x = 36$ (Darlington & Wylie, 1955; Mehra & Sachdeva, 1976), *K. pygmaea* was suggested to be di- to octaploid, with irregularities arising from agmato- and aneuploidy. Based on an extensive and comparable dataset, we demonstrated that *K. pygmaea* evidently has a tetraploid chromosome set with $2n = 4x = 64$. For the other species, some caution is required, because our counts and SSR analyses are only directly comparable for those four species for which tissues were retrieved from the same individual. In any case, chromosome numbers of all species were in accordance with the maximum number of alleles, indicating the two basic numbers $x = 16$ and 18. Further support comes from the fact that individuals with 16 and 18 chromosomes have not yet been found. The lowest ever published chromosome numbers in the genus are $2n = 32$ in *K. schoenoides* (Poghosian *et al*., 1971) and $2n = c.\ 36$ in *K. myosuroides* (reviewed in Aiken *et al*., 2007b), which correspond to diploids rather than tetraploids.

Tischler (1950) counted $n = 26$ for haploid tissue of *K. myosuroides*. The resulting diploid chromosome number $2n = 52$ is not a multiple of $x = 16$ or $x = 18$, and thus $x = 26$ may represent a third basic number. Chromosome numbers of *K. curvata* ($2n = 50$, Yano *et al*., 2011) and *K. duthiei* ($2n = c.\ 84$, Yano *et al*., 2011) could represent multiples (Table 2). In any case, *K. myosuroides* is the species with the most variable chromosome counts ranging from $2n = 4x = 52$ to 66. With our approach we cannot clarify which chromosome number is the primary one, and which arose through agmatoploidy or aneuploidy. Estimates of possible basic numbers and derived ploidy thus remain speculative (Table 2).

The suggested basic numbers are multiples of $x = 8$, 9 and 13 (Mehra & Sachdeva, 1976). Thus, *Kobresia* spp. may represent palaeopolyploids: ancient polyploids forming quadrivalents at meiosis, which became diploid again, having two pairs of chromosomes, each of which formed bivalents. This mechanism is common in evolution and in the speciation of fungi, plants including angiosperms and animals including mammals (Wolfe, 2001). Cytological studies in autotetraploid *Carex multifolia* Ohwi revealed bivalent rather than tetravalent chromosome associations (Tanaka, 1940a), and palaeopolyploids are known from other genera of the Himalayan region (Yuan *et al*., 1998).

However, recent polyploidization is also widespread in *Kobresia*, with duplication of the entire chromosome set being a driving factor for speciation (Ramsey & Schemske, 1998; Soltis *et al*., 2004). With more than one-half of the tested species being polyploid, the rate is high compared with 5.7% in the closely related genus *Carex* (Lipnerová *et al*., 2013). In *Carex*, ploidy is hardly associated with speciation (Löve, Löve & Raymond, 1957; Tang & Xiang, 1989; Hipp *et al*., 2009b). The same holds true for most other genera of Cyperaceae, including *Cyperus*, *Eriophorum* L. and *Scirpus*. Exceptions include, for instance, the genera *Schoenoplectus*, *Rhynchospora* Vahl and *Eleocharis* R.Br., in which a multimodal distribution of haploid chromosome numbers indicates multiple ploidies, in some cases associated with subsequent aneuploidy (Roalson, 2008).

The origin of ploidy in Cyperaceae is still subject to discussion. The most common mechanism in angiosperms is via the formation of unreduced gametes. The production of such gametes is heritable (Parisod, Holderegger & Brochmann, 2010), but considered to be rare in Cyperaceae because of the abortion of three nuclei after meiosis, resulting in the formation of pseudomonad pollen instead of tetrads (Heilborn, 1934; but see Hoshino & Okamura, 1994). However, environmental stress, such as frost, wounding, herbivory, water deficit or low amounts of nutrients, increases the rate of production of unreduced gametes (Ramsey & Schemske, 1998; Parisod *et al*., 2010). Another supposed mechanism of autopolyploid formation is somatic doubling, which has been suggested for *Carex glauca* Scop. (Heilborn, 1934), *C. sidero-sticta* Hance (Tanaka, 1940b) and *C. multifolia* (Tanaka, 1940a).

Here, we showed that adapting basic numbers from the divisor of chromosome numbers may yield a misleading picture of ploidy, in which ancient polyploids diploidized or haploid numbers vary in the range of the basic number because of cytological abnormalities. As such, in Cyperaceae, neither chromosome counts nor analyses of the maximum number of alleles alone can reliably determine basic chromosome numbers or, consequently, ploidy. Inconsistencies between the maximum number of alleles and the suggested basic numbers have also been observed in other species (Bousalem *et al*., 2006) and will increase

further with increasing use of co-dominant markers. Thus, the verification of ploidy in all taxa, as opposed to current assumptions based exclusively on one of the three methods, will be both worthwhile and feasible in the future.

## CONCLUSION

Ploidy has been a major factor in the evolution of *Kobresia*, in ancient times and more recently, in which it may facilitate ecological adaptability (Hagerup, 1932; Stebbins, 1950) to the extreme conditions of its alpine distribution range.

Our combined analyses led us to the new conclusion that *Kobresia* has at least two basic numbers at $x = 16$ and 18, with ploidy ranging from diploid to heptaploid, and *K. pygmaea* is tetraploid. This is a further step towards gaining a full understanding of the phylogenetics and chromosomal evolution in this taxon.

## ACKNOWLEDGEMENTS

We thank Birgit Müller, Leane Boerner and Stefan Michalski for their valuable assistance in laboratory work. Plant material was kindly provided by Sabine and Georg Miehe (University Marburg, High Asia Project Herbarium) and by the staff from the 'Brockengarten' (Harz National Park, Germany); special thanks go to Gunter Karste for providing living plants and thus enabling our own chromosome counts. Christiane Ritz and Andrew Davis made helpful comments on an earlier version of the manuscript; the final version benefitted much from suggestions by two anonymous referees. This research was funded by the German Science Foundation as part of the DFG Priority Programme 'TiP–Tibetan Plateau: Formation, Climate, Ecosystems (SPP 1372)' (Research Grants WE 2601/4-1 and WE 2601/4-2 to KW) and the Key Project of International Collaboration Program from the Ministry of Science and Technology of China (Research Grant 2010DFB63500 to JL). The authors declare that they have no further conflicts of interest.

## REFERENCES

**Aiken SG, Dallwitz MJ, Consaul LL, McJannet CL, Boles RL, Argus GW, Gillett MJ, Scott PJ, Elven R, LeBlanc MC, Gillespie LJ, Brysting AK, Solstad H, Harris JG. 2007a.** Flora of the Canadian Arctic Archipelago: descriptions, illustrations, identification, and information retrieval. Ottawa: NRC Research Press, National Research Council of Canada. Available at http://nature.ca/aaflora/data/www/cykosb.htm (accessed 15 August 2014).

**Aiken SG, Dallwitz MJ, Consaul LL, McJannet CL, Boles RL, Argus GW, Gillett MJ, Scott PJ, Elven R, LeBlanc MC, Gillespie LJ, Brysting AK, Solstad H, Harris JG. 2007b.** Flora of the Canadian Arctic Archipelago: descriptions, illustrations, identification, and information retrieval. Ottawa: NRC Research Press, National Research Council of Canada. Available at http://nature.ca/aaflora/data/www/cykomy.htm (accessed 15 August 2014).

**Avolio ML, Beaulieu JM, Lo EYY, Smith MD. 2012.** Measuring genetic diversity in ecological studies. *Plant Ecology* **213:** 1105–1115.

**Baack EJ. 2004.** Cytotype segregation on regional and microgeographic scales in snow buttercups (*Ranunculus adoneus*: Ranunculaceae). *American Journal of Botany* **91:** 1783–1788.

**Barbará T, Palma-Silva C, Paggi GM, Bered F, Fay MF, Lexer C. 2007.** Cross-species transfer of nuclear microsatellite markers: potential and limitations. *Molecular Ecology* **16:** 3759–3767.

**Besnard G, Garcia-Verdugo C, Rubio De Casas R, Treier UA, Galland N, Vargas P. 2008.** Polyploidy in the olive complex (*Olea europaea*): evidence from flow cytometry and nuclear microsatellite analyses. *Annals of Botany* **101:** 25–30.

**Blum MJ, McLachlan JS, Saunders CJ, Herrick JD. 2005.** Characterization of microsatellite loci in *Schoenoplectus americanus* (Cyperaceae). *Molecular Ecology Notes* **5:** 661–663.

**Bousalem M, Arnau G, Hochu I, Arnolin R, Viader V, Santoni S, David J. 2006.** Microsatellite segregation analysis and cytogenetic evidence for tetrasomic inheritance in the American yam *Dioscorea trifida* and a new basic chromosome number in the Dioscoreae. *Theoretical and Applied Genetics* **113:** 439–451.

**Brochmann C, Soltis PS, Soltis DE. 1992.** Recurrent formation and polyphyly of Nordic polyploids in *Draba* (Brassicaceae). *American Journal of Botany* **79:** 673–688.

**Burnier J, Buerki S, Arrigo N, Küpfer P, Alvarez N. 2009.** Genetic structure and evolution of Alpine polyploid complexes: *Ranunculus kuepferi* (Ranunculaceae) as a case study. *Molecular Ecology* **18:** 3730–3744.

**Clark LV, Jasieniuk M. 2011.** POLYSAT: an R package for polyploid microsatellite analysis. *Molecular Ecology Resources* **11:** 562–566.

**Collada C, Fuentes-Utrilla P, Gil L, Cervera MT. 2004.** Characterization of microsatellite loci in *Ulmus minor* Miller and cross-amplification in *U. glabra* Hudson and *U. laevis* Pall. *Molecular Ecology Notes* **4:** 731–732.

**Cui XK, Ao CQ, Zhang Q, Chen LT, Liu JQ. 2008.** Diploid and tetraploid distribution of *Allium przewalskianum* Regel (Liliaceae) in the Qinghai–Tibetan Plateau and adjacent regions. *Caryologia* **61:** 1–9.

**Dahlgren R, Clifford HT, Yeo P. 1985.** *The families of the monocotyledons: structure, evolution, and taxonomy*. Berlin, New York: Springer-Verlag.

**Darlington CD, Wylie AP. 1955.** *Chromosome atlas of flowering plants*. London: George Allen & Unwin Ltd.

**Davies EW. 1956.** Cytology, evolution and origin of the aneuploid series in the genus *Carex*. *Hereditas* **42:** 349–365.

**Doležel J, Greilhuber J, Suda J. 2007.** Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols* **2:** 2233–2244.

**Doyle JJ, Doyle J. 1987.** A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin, Botanical Society of America* **19:** 11–15.

**Elven R. 2007.** Checklist of the Panarctic Flora (PAF) vascular plants. Version: May 2007. Available at http://nhm2.uio.no/paf/results?biogeographic=&bioclimatic=&region=&name=kobresia (accessed 15 August 2014).

**Erler A, Stoneking M, Kayser M. 2004.** Development of Y-chromosomal microsatellite markers for nonhuman primates. *Molecular Ecology* **13:** 2921–2930.

**Esselink GD, Nybom H, Vosman B. 2004.** Assignment of allelic configuration in polyploids using the MAC-PR (microsatellite DNA allele counting – peak ratios) method. *Theoretical and Applied Genetics* **109:** 402–408.

**Faircloth BC. 2008.** MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology* **8:** 92–94.

**Gauthier P, Lumaret R, Bédécarrats A. 1998.** Genetic variation and gene flow in Alpine diploid and tetraploid populations of *Lotus* (*L. alpinus* (D.C.) Schleicher/*L. corniculatus* L.). I. Insights from morphological and allozyme markers. *Heredity* **80:** 683–693.

**Goldman DH, Jansen RK, van den Berg C, Leitch IJ, Fay MF, Chase MW. 2004.** Molecular and cytological examination of *Calopogon* (Orchidaceae, Epidendroideae): circumscription, phylogeny, polyploidy, and possible hybrid speciation. *American Journal of Botany* **91:** 707–723.

**Grant V. 1981.** *Plant speciation*. New York: Columbia University Press.

**Hagerup O. 1932.** Über Polyploidie in Beziehung zu Klima, Ökologie und Phylogenie. *Hereditas* **16:** 19–40.

**Heilborn O. 1924.** Chromosome numbers and dimensions, species-formation and phylogeny in the genus *Carex*. *Hereditas* **5:** 129–216.

**Heilborn O. 1934.** On the origin and preservation of polyploidy. *Hereditas* **19:** 233–242.

**Heilborn O. 1939.** Chromosome studies in Cyperaceæ III–IV. *Hereditas* **25:** 224–240.

**Hijmans RJ, Gavrilenko T, Stephenson S, Bamberg J, Salas A, Spooner DM. 2007.** Geographical and environmental range expansion through polyploidy in wild potatoes (*Solanum* section *Petota*). *Global Ecology and Biogeography* **16:** 485–495.

**Hipp AL, Kettenring KM, Feldheim KA, Weber JA. 2009a.** Isolation of 11 polymorphic tri- and tetranucleotide microsatellite loci in a North American sedge (*Carex scoparia*: Cyperaceae) and cross-species amplification in three additional *Carex* species. *Molecular Ecology Resources* **9:** 625–627.

**Hipp AL, Rothrock PE, Reznicek AA, Berry PE. 2007.** Chromosome number changes associated with speciation in sedges: a phylogenetic study in *Carex* section *Ovales* (Cyperaceae) using AFLP data. *Aliso* **23:** 193–203.

**Hipp AL, Rothrock PE, Roalson EH. 2009b.** The evolution of chromosome arrangements in *Carex* (Cyperaceae). *The Botanical Review* **75:** 96–109.

**Hoshino T, Okamura K. 1994.** Cytological studies on meiotic configurations of intraspecific aneuploids of *Carex blepharicarpa* (Cyperaceae) in Japan. *Journal of Plant Research* **107:** 1–8.

**Hoshino T, Rajbhandari KR, Ohba H. 2000.** Cytological studies of eleven species of Cyperaceae collected from central Nepal. *Cytologia* **65:** 219–224.

**Jäger EJ, Werner K, eds. 2002.** *Exkursionsflora von Deutschland: Gefäßpflanzen: Grundband*. Berlin: Spektrum Akademischer Verlag Heidelberg.

**Knaben G, Engelskjøn T. 1967.** Chromosome numbers of Scandinavian arctic–alpine plant species II. *Acta Borealia A. Scientia* **21:** 1–57.

**Leck MA, Schütz W. 2005.** Regeneration of Cyperaceae, with particular reference to seed ecology and seed banks. *Perspectives in Plant Ecology, Evolution and Systematics* **7:** 95–133.

**Lipnerová I, Bureš P, Horová L, Šmarda P. 2013.** Evolution of genome size in *Carex* (Cyperaceae) in relation to chromosome number and genomic base composition. *Annals of Botany* **111:** 79–94.

**Liu WS, Dong M, Song ZP, Wei W. 2009.** Genetic diversity pattern of *Stipa purpurea* populations in the hinterland of Qinghai–Tibet Plateau. *Annals of Applied Biology* **154:** 57–65.

**Löve Á, Löve D, Raymond M. 1957.** Cytotaxonomy of *Carex* section *Capillares*. *Canadian Journal of Botany* **35:** 715–761.

**Luceño M. 1994.** Cytotaxonomic studies in Iberian, Balearic, North African, and Macaronesian species of *Carex* (Cyperaceae): II. *Canadian Journal of Botany* **72:** 587–596.

**Luceño M, Castroviejo S. 1991.** Agmatoploidy in *Carex laevigata* (Cyperaceae). Fusion and fission of chromosomes as the mechanism of cytogenetic evolution in Iberian populations. *Plant Systematics and Evolution* **177:** 149–159.

**Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer MLI, Jarvie TP, Jirage KB, Kim J-B, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu P, Begley RF, Rothberg JM. 2005.** Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437:** 376–380.

**Mehra PN, Sachdeva SK. 1976.** Cytology of some sedges from north west India. *Cytologia* **41:** 585–590.

**Meng Y, Sun H, Yang YP, Nie ZL. 2010.** Polyploidy and new chromosome counts in *Anaphalis* (Asteraceae: Gnaphalieae) from the Qinghai–Tibet Plateau of China. *Journal of Systematics and Evolution* **48:** 58–64.

**Miehe G, Miehe S, Bach K, Nölling J, Hanspach J, Reudenbach C, Kaiser K, Wesche K, Mosbrugger V, Yang YP, Ma YM. 2011.** Plant communities of central

Tibetan pastures in the Alpine Steppe/*Kobresia pygmaea* ecotone. *Journal of Arid Environments* **75:** 711–723.

**Miehe G, Miehe S, Kaiser K, Liu J, Zhao X. 2008.** Status and dynamics of the *Kobresia pygmaea* ecosystem on the Tibetan Plateau. *AMBIO: A Journal of the Human Environment* **37:** 272–279.

**Otto F. 1990.** DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Darzynkiewicz Z, Crissman HA, eds. *Methods in cell biology, vol. 33: flow cytometry*. New York: Academic Press, 105–110.

**Parisod C, Besnard G. 2007.** Glacial *in situ* survival in the Western Alps and polytopic autopolyploidy in *Biscutella laevigata* L.(Brassicaceae). *Molecular Ecology* **16:** 2755–2767.

**Parisod C, Holderegger R, Brochmann C. 2010.** Evolutionary consequences of autopolyploidy. *New Phytologist* **186:** 5–17.

**Peakall R, Gilmore S, Keys W, Morgante M, Rafalski A. 1998.** Cross-species amplification of soybean (*Glycine max*) simple sequence repeats (SSRs) within the genus and other legume genera: implications for the transferability of SSRs in plants. *Molecular Biology and Evolution* **15:** 1275–1287.

**Pellicer J, Clermont S, Houston L, Rich TCG, Fay MF. 2012.** Cytotype diversity in the *Sorbus* complex (Rosaceae) in Britain: sorting out the puzzle. *Annals of Botany* **110:** 1185–1193.

**Pfeiffer T, Roschanski AM, Pannell JR, Korbecka G, Schnittler M. 2011.** Characterization of microsatellite loci and reliable genotyping in a polyploid plant, *Mercurialis perennis* (Euphorbiaceae). *Journal of Heredity* **102:** 479–488.

**Poghosian AJ, Narinian SG, Voskanian VE. 1971.** Data on the caryogeographical study of flora of the Aragatz massif. *Biologicheskii Zhurnal Armenii* **24:** 37–43.

**Probatova NS, Sokolovskaya AP. 1988.** Chromosome numbers in vascular plants from Primorye Territory, the Amur River basin, north Koryakia, Kamchatka and Sakhalin. *Botanicheskii Zhurnal* **73:** 290–293.

**Ramsey J, Schemske DW. 1998.** Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics* **29:** 467–501.

**Ranker TA, Haufler CH, Soltis PS, Soltis DE. 1989.** Genetic evidence for allopolyploidy in the neotropical fern *Hemionitis pinnatifida* (Adiantaceae) and the reconstruction of an ancestral genome. *Systematic Botany* **14:** 439–447.

**Ritz CM, Wissemann V. 2011.** Microsatellite analyses of artificial and spontaneous dogrose hybrids reveal the hybridogenic origin of *Rosa micrantha* by the contribution of unreduced gametes. *Journal of Heredity* **102:** 217–227.

**Roalson EH. 2008.** A synopsis of chromosome number variation in the Cyperaceae. *The Botanical Review* **74:** 209–393.

**Roalson EH, Columbus JT, Friar EA. 2001.** Phylogenetic relationships in Cariceae (Cyperaceae) based on ITS (nrDNA) and *trnT-LF* (cpDNA) region sequences: assessment of subgeneric and sectional relationships in *Carex* with emphasis on section *Acrocystis*. *Systematic Botany* **26:** 318–341.

**Rozen S, Skaletsky H. 1999.** Primer3 on the www for general users and for biologist programmers. In: Misener S, Krawetz SA, eds. *Methods in molecular biology, vol. 132: bioinformatics methods and protocols*. Totowa, NJ: Humana Press Inc., 365–386.

**Schönswetter P, Lachmayer M, Lettner C, Prehsler D, Rechnitzer S, Reich DS, Sonnleitner M, Wagner I, Hülber K, Schneeweiss GM, Trávníček P, Suda J. 2007.** Sympatric diploid and hexaploid cytotypes of *Senecio carniolicus* (Asteraceae) in the Eastern Alps are separated along an altitudinal gradient. *Journal of Plant Research* **120:** 721–725.

**Schuelke M. 2000.** An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* **18:** 233–234.

**Schwarzacher T, Ambros P, Schweizer D. 1980.** Application of Giemsa banding to orchid karyotype analysis. *Plant Systematics and Evolution* **134:** 293–297.

**Soltis DE, Rieseberg LH. 1986.** Autopolyploidy in *Tolmiea menziesii* (Saxifragaceae): genetic insights from enzyme electrophoresis. *American Journal of Botany* **73:** 310–318.

**Soltis DE, Soltis PS. 1995.** The dynamic nature of polyploid genomes. *Proceedings of the National Academy of Sciences of the United States of America* **92:** 8089–8091.

**Soltis DE, Soltis PS. 1999.** Polyploidy: recurrent formation and genome evolution. *Trends in Ecology & Evolution* **14:** 348–352.

**Soltis DE, Soltis PS, Tate JA. 2004.** Advances in the study of polyploidy since plant speciation. *New Phytologist* **161:** 173–191.

**Stark C, Michalski SG, Babik W, Winterfeld G, Durka W. 2011.** Strong genetic differentiation between *Gymnadenia conopsea* and *G. densiflora* despite morphological similarity. *Plant Systematics* **293:** 213–226.

**Stebbins GL. 1950.** *Variation and evolution in plants*. New York: Columbia University Press.

**Stebbins GL. 1985.** Polyploidy, hybridization, and the invasion of new habitats. *Annals of the Missouri Botanical Garden* **72:** 824–832.

**Suda J, Trávníček P. 2006.** Reliable DNA ploidy determination in dehydrated tissues of vascular plants by DAPI flow cytometry – new prospects for plant research. *Cytometry Part A* **69A:** 273–280.

**Tanaka N. 1940a.** Chromosome studies in Cyperaceae, X: aneuploid plants of *Carex multifolia* Ohwi. *Botanic Magazine (Tokyo)* **54:** 438–446.

**Tanaka N. 1940b.** Chromosome studies in Cyperaceae, VIII: meiosis in diploid and tetraploid forms of *Carex siderosticta* Hance. *Cytologia* **11:** 282–310.

**Tang YC, Xiang QY. 1989.** Cytological studies of *Carex siderostricta* Hance (Cyperaceae) and its importance in phytogeography. *Cathaya* **1:** 49–60.

**Thrall P, Young A. 2000.** Computer note: AUTOTET: a program for analysis of autotetraploid genotypic data. *The Journal of Heredity* **91:** 348–349.

**Tischler GFL. 1950.** *Die Chromosomenzahlen der Gefässpflanzen Mitteleuropas*. 's-Gravenhage: W Junk.

**Wahl HA. 1940.** Chromosome numbers and meiosis in the genus *Carex*. *American Journal of Botany* **27:** 458–470.

**Waterway MJ. 1994.** Evidence for the hybrid origin of *Carex knieskernii* with comments on hybridization in the genus *Carex* (Cyperaceae). *Canadian Journal of Botany* **72:** 860–871.

**Winterfeld G, Röser M. 2007.** Chromosomal localization and evolution of satellite DNAs and heterochromatin in grasses (Poaceae), especially tribe Aveneae. *Plant Systematics and Evolution* **264:** 75–100.

**Wolfe KH. 2001.** Yesterday's polyploids and the mystery of diploidization. *Nature Reviews Genetics* **2:** 333–341.

**Wood TE, Takebayashi N, Barker MS, Mayrose I, Greenspoon PB, Rieseberg LH. 2009.** The frequency of polyploid speciation in vascular plants. *Proceedings of the National Academy of Sciences of the United States of America* **106:** 13 875–13 879.

**Wu LL, Cui XK, Milne RI, Sun YS, Liu JQ. 2010.** Multiple autopolyploidizations and range expansion of *Allium przewalskianum* Regel. (Alliaceae) in the Qinghai–Tibetan Plateau. *Molecular Ecology* **19:** 1691–1704.

**Wyatt R, Odrzykoski IJ, Stoneburner A, Bass HW, Galau GA. 1988.** Allopolyploidy in bryophytes: multiple origins of *Plagiomnium medium*. *Proceedings of the National Academy of Sciences of the United States of America* **85:** 5601–5604.

**Yamane K, Yasui Y, Ohnishi O. 2003.** Intraspecific cpDNA variations of diploid and tetraploid perennial buckwheat, *Fagopyrum cymosum* (Polygonaceae). *American Journal of Botany* **90:** 339–346.

**Yano O, Ikeda H, Watson MF, Rajbhandari KR. 2011.** Cytological studies of 4 species of the genus *Kobresia* (Cyperaceae) collected from Nepal Himalaya. *Cytologia* **76:** 137–141.

**Yuan YM, Küpfer P, Zeltner L. 1998.** Chromosomal evolution of *Gentiana* and *Jaeschkea* (Gentianaceae), with further documentation of chromosome data for 35 species from western China. *Plant Systematics and Evolution* **210:** 231–247.

**Zhang SR. 2001.** A preliminary revision of the supraspecific classification of *Kobresia* Willd. (Cyperaceae). *Botanical Journal of the Linnean Society* **135:** 289–294.

**Zhang SR, Liang SY, Dai LK. 1995.** A study on the geographic distribution of the genus *Kobresia* Willd. *Acta Phytotaxonomica Sinica* **33:** 144–160.

**Zhang SR, Noltie HJ. 2010.** *Kobresia* Willdenow, Sp. Pl. 4: 205. 1805. *Flora of China* **23:** 269–285.

**Zhao QF, Wang G, Li QX, Ma SR, Cui Y, Grillo M. 2006.** Genetic diversity of five *Kobresia* species along the eastern Qinghai–Tibet plateau in China. *Hereditas* **143:** 33–40.

**Zhou Y, Wang H, Yang M, Chen J, Zhang W. 2009.** Development of microsatellites for *Scirpus mariqueter* Wang et Tang (Cyperaceae) and cross-species amplification in *Scirpus planiculmis* F. Schmidt. *Molecular Ecology Resources* **9:** 370–372.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Results of the flow cytometric measurements.

**Table S1.** Sources of studied *Kobresia* specimens.

**Table S2.** Characteristics of 12 additionally tested microsatellite loci in tetraploid *Kobresia pygmaea*. The sequence of the forward primer represents a fusion of a universal tag sequence (CAG-tag: 5′-CAG TCG GGC GTC ATC A-3′; M13(-21)-tag: 5′-TGT AAA ACG ACG GCC AGT-3′) and the selective primer sequence. The tag sequence is highlighted; the expected size is given including the tag. GenBank accession numbers refer to the DNA Data Bank of Japan (DDBJ).

**Table S3.** Characteristics of 161 microsatellite loci in tetraploid *Kobresia pygmaea*. The primers were identified by Primer3 (Rozen & Skaletsky, 1999), but have not been tested. The sequence of the forward primer represents a fusion of a universal tag sequence (CAG-tag: 5′-CAG TCG GGC GTC ATC A-3′; M13(-21)-tag: 5′-TGT AAA ACG ACG GCC AGT- 3′) and the selective primer sequence. The tag sequence is highlighted; the expected size is given including the tag. GenBank accession numbers refer to the DNA Data Bank of Japan (DDBJ).

**Table S4.** Performance of eight microsatellite markers in *K. pygmaea* and other species of the genus *Kobresia*.