MOLECULAR ECOLOGY

Molecular Ecology (2015) **24**, 4023–4038 doi: 10.1111/mec.13278

Plant–herbivorous beetle networks: molecular characterization of trophic ecology within a threatened steppic environment

Ł. KAJTOCH,* D. KUBISZ,* W. HEISE,† M. A. MAZUR‡ and W. BABIK§

*Institute of Systematics and Evolution of Animals Polish Academy of Sciences, Sławkowska 17, 31-016, Krakow, Poland, †Centre for Ecology and Hydrology, ECW, Deiniol Road, Bangor LL57 2UW, UK, ‡Center for Biodiversity Studies, Department of Biosystematics, Opole University, Opole, Poland, §Institute of Environmental Sciences, Jagiellonian University, Krakow, Poland

Abstract

DNA barcoding facilitates many evolutionary and ecological studies, including the examination of the dietary diversity of herbivores. In this study, we present a survey of ecological associations between herbivorous beetles and host plants from seriously threatened European steppic grasslands. We determined host plants for the majority (65%) of steppic leaf beetles (55 species) and weevils (59) known from central Europe using two barcodes (trnL and rbcL) and two sequencing strategies (Sanger for mono/oligophagous species and Illumina for polyphagous taxa). To better understand the ecological associations between steppic beetles and their host plants, we tested the hypothesis that leaf beetles and weevils differ in food selection as a result of their phylogenetic relations (within genera and between families) and interactions with host plants. We found 224 links between the beetles and the plants. Beetles belonging to seven genera feed on the same or related plants. Their preferences were probably inherited from common ancestors and/or resulted from the host plant's chemistry. Beetles from four genera feed on different plants, possibly reducing intrageneric competition and possibly due to an adaptation to different plant chemical defences. We found significant correlations between the numbers of leaf beetle and weevil species feeding on particular plants for polyphagous taxa, but not for nonpolyphagous beetles. Finally, we found that the previous identifications of host plants based on direct observations are generally concordant with host plant barcoding from insect gut. Our results expand basic knowledge about the trophic relations of steppic beetles and plants and are immediately useful for conservation purposes.

Keywords: Chrysomelidae, Curculionidae, DNA barcoding, host plant, molecular ecology, xerothermic grasslands

Received 10 January 2015; revision received 12 June 2015; accepted 15 June 2015

Introduction

The recent development of the concept of barcoding enables examined specimens to be assigned to the appropriate species relatively simply and quickly (Hebert *et al.* 2003; Moritz & Cicero 2004; Pons *et al.* 2006). It also provides an opportunity for identifying the DNA of other organisms present inside the bodies of the examined specimens (Valentini *et al.* 2009a,b; Taberlet *et al.* 2012). This could facilitate many evolutionary and ecological studies, such as the examination of the dietary diversity of predators, fungi-eaters and herbivores (Symondson 2002; Harper *et al.* 2005; Sheppard & Harwood 2005; Valentini *et al.* 2009a,b; Taberlet *et al.* 2012). Previous methods used for the study of herbivores included the direct observation of feeding animals (Sandholm & Price 1962; Dieckmann 1980; Barone

Correspondence: Łukasz Kajtoch, Fax: +48 12 422-42-94; E-mail: lukasz.kajtoch@gmail.com

1998; Novotny *et al.* 2002, 2006; Dyer *et al.* 2007) and the analysis of faeces (Holechek *et al.* 1982; Johnson & Nicolson 2001) or gut content (Otte & Joern 1976; Fry *et al.* 1978) using morphological or chemical approaches (Dove & Mayes 1996; Dahle *et al.* 1998; Foley *et al.* 1998). All of these methods have serious limitations with regard to their discriminatory power, as they rarely allow for the identification of host plants at the species level. In addition, these methods are time-consuming.

In the last few years, significant progress has been made in the barcoding of associations between host plants and insects (Matheson *et al.* 2008). The majority of the pioneering studies in this field were performed on Coleoptera (Jurado-Rivera *et al.* 2009; Pinzón-Navarro *et al.* 2010; Garcia-Robledo *et al.* 2013; Kishimoto *et al.* 2013; Kitson *et al.* 2013) and Orthoptera (Ibanez *et al.* 2013; Avanesyan 2014). Interactions between herbivorous beetles and flowering plants have been postulated as major drivers of beetle diversity (Farrell 1998), as 135 000 of 360 000 beetle species are phytophagous (Gillot 2005; Zhi-Quiang 2013). So far, all host plant barcoding studies on beetles have been performed on the two most speciose groups: weevils (Curculionoidea; >62 000 known species, Oberprieler *et al.* 2007) and leaf beetles (Chrysomelidae; around 35 000 known species; Jolivet & Verma 2002). It is not surprising that all of these studies focused on species associated with tropical forests (Jurado-Rivera *et al.* 2009; Pinzón-Navarro *et al.* 2010; Garcia-Robledo *et al.* 2013; Kishimoto *et al.* 2013; Kitson *et al.* 2013), as interactions between tropical insects and plants have been a target of many other studies, due to the extremely high diversity of both tropical plants and insects (e.g. Novotny *et al.* 2002, 2006, 2007). Similar studies should be performed in other areas and habitats, particularly those that sustain diverse assemblages of plants and herbivores, to expand our knowledge of the evolutionary interactions and ecological associations between herbivores and plants. The results of such studies could also be very valuable for conservation purposes in threatened environments. Previous studies have often had limitations as they were performed (i) on beetle samples collected from traps, highly reducing the success of barcode amplification, or (ii) without the development of a barcode database for local flora, which often limited identification to the family or genus level (Jurado-Rivera *et al.* 2009; Pinzón-Navarro *et al.* 2010; Kishimoto *et al.* 2013). Most of these studies also used single individuals for the identification of host plants, which could be problematic in polyphagous taxa (see Kajtoch 2014).

In this study, we focused on plant and beetle assemblages of steppic habitats – xerothermic grasslands from central Europe with an extrazonal threatened plant community closely related to the Eurasian steppes. An essential initial step of our study was to evaluate, using barcoding data, the accuracy of inferences about the feeding preferences of beetles based on direct observations as described in the literature (Szymczakowski 1960; Warchałowski 1991; Mazur 2001). The primary aim of this study was to test hypotheses that could explain the ecological associations between herbivores and their host plants. We compared the diet of beetles on two taxonomic levels: interfamily (weevils vs. leaf beetles) and intrageneric. The purpose of a comparison on the family level was to examine whether these two exophagous (as imago) groups of beetles differed in food selection (Mitter & Farrell 1991; Farrell 1998). We addressed this considering separately polyphagous taxa and nonpolyphagous taxa to test the hypothesis that differences in food selection are dependent on feeding specialization. In other words, our hypothesis was that mono/oligophagous weevils and leaf beetles utilize different host plants as they consume plants selectively, but polyphagous beetles tend to favour similar plants (Bernays & Chapman 1994; Jolivet 1998). Intrageneric comparisons test the hypothesis that phylogenetically related species feed on the same or related host plants. We discuss these hypotheses in the context of macroevolutionary scenarios of insect–plant interactions (Jermy 1976, 1984; Futuyma & Mitter 1996; Janz *et al.* 2006; Agrawal 2007): co-evolution, competition for food resources and natural selection to improve insects' ability to deal with host plant chemical defences (Ehrlich & Raven 1964; Schultz 1988; Becerra 1997; Hartley & Jones 1997; Becerra & Venable 1999). Finally, our results are discussed in the light of their relevance for the conservation of declining populations and the management of rare and threatened steppic habitats.

Methods

Sampling sites and the development of a plant barcode database

The sampling was performed in the steppic (xerothermic, calcareous) grasslands of *Festuco-Brometea* phytocoenoses located in central Europe. This region sustains a network of relatively well-preserved steppic habitats with communities rich in plant and insect species, including very diverse assemblages of beetles (e.g. Mazur 2001, 2002; Wąsowska 2006; Mazur & Kubisz 2013).

This type of habitat was chosen for several reasons: (i) we have a good knowledge of steppic plants and beetles and their communities, as they have already been intensively studied in central Europe (e.g. Preuss 1912; Kuntze 1931; Szymczakowski 1960, 1965;

Ceynowa 1968; Warchałowski 1976; Mazur 2001, 2006; Wąsowska 2006; Chytrý 2007; Nazarenko 2009; Mazur & Kubisz 2013), (ii) it contains all major types of steppic grasslands and associated species-rich communities of plants and beetles in central Europe (Mazur 2001, 2002; Zając & Zając 2001; Matuszkiewicz 2005; Mazur & Kubisz 2013), (iii) it has a high level of threat and conservation needs – many steppic species are rare, threatened or even endangered (Binot *et al.* 1998; Holecová & Franc 2001; Pawłowski *et al.* 2002; Farkač *et al.* 2005), (iv) there is an availability of data about the diet of steppic beetles – some of them have been studied, but only on the basis of direct observations (e.g. Szymczakowski 1960; Warchałowski 1991; Mazur 2001), and (v) a multilocus database of barcodes for steppic plants from central Europe has recently been developed (Heise *et al.* 2015), allowing for the direct, accurate and efficient identification of host plants.

A database of plant barcodes (trnL, rbcL and matK) was developed in 2014 on the basis of steppic (xerothermic) plant sampling in Poland (Heise *et al.* 2015). The database includes trnL and rbcL sequences for 128 plant species and matK sequences for 115 plant species, constituting approximately 85% of the steppic plant species from central Europe.

Beetle sampling

The target selected for this study was two groups of beetles: weevils (Curculionoidea: Anthribidae, Apionidae and Curculionidae) and leaf beetles (Chrysomelidae). These are most species rich in steppic habitats and were objects of many previous studies, both classical zoogeographical and ecological (e.g. Mazur 2001, 2002; Wąsowska 2006; Mazur & Kubisz 2013) as well as phylogeographic (e.g. Kajtoch *et al.* 2013; Kubisz *et al.* 2012; Mazur *et al.* 2014). There are around 114 known weevil species associated with steppic grasslands in Poland (approximately 11% of all weevils in the country; Mazur 2001; Wanat & Mokrzycki 2005) and 85 leaf beetle species inhabiting this environment (approximately 17% of all leaf beetles in the country; Borowiec *et al.* 2011). The majority of steppic beetles are either known or assumed to feed on a few related species from a single family, on a single plant species or closely related members of the same genus, whereas less than a quarter of species feed on diverse plants from different taxonomic groups.

We aimed to only sample beetle species known to inhabit the steppic grasslands of southern Poland (where the majority of plant species were collected for the barcode database development). Therefore, the majority (>90%) of the beetle species were collected in southern Poland (in the uplands localized between the cities of Kraków and Kielce; coordinates of the centre of this area are 50.374°N and 20.407°E). Some beetle species which could not be found due to their rarity in southern Poland or because their populations are extinct in this region were collected in the neighbouring regions of central and eastern central Europe (in Moravia in the Czech Republic, southern Slovakia, northern Hungary and Podolia in western Ukraine; see Data accessibility). Beetles were collected in sweep nets during several field trips in May and June 2011–2014. Beetles were only collected in good weather conditions to avoid collecting starving specimens, as the efficiency of plant DNA isolation and amplification is decreased in starving individuals (Kajtoch & Mazur 2015). The specimens were then immediately preserved in the field in ethanol (96%) to minimize DNA degradation. Samples were kept frozen until DNA isolation. Due to the rarity of most of the examined species, only 1–2 specimens could be collected and used for barcoding. For several species, especially those known to be polyphagous and for which we were able to collect at least 10 specimens, preferably each from a different locality, a larger number of specimens (10–16) were analysed (details in Table 1).

Laboratory procedures

Whole beetles were digested with proteinase K, and DNA was isolated using a Sherlock AX kit (A&A Biotechnology) dedicated to the isolation of DNA traces from low-quality samples. The DNA concentration and purity of all isolates was assessed using Nanodrop. In addition, the quality of the DNA isolates from the beetles was checked by amplifying the COI mitochondrial gene using primers that have frequently been used in other studies on beetles (C1-J- 2183 and TL2-N-3014; Simon *et al.* 1994). These sequences were also used in further phylogenetic analyses (see below). Next, DNA isolates were used for the amplification of two chloroplast barcodes, that is the rbcL gene and the trnL intron, using the following primers: rbcL-F1 and rbcL-724R (Fay *et al.* 1997), and A49325 and B49863 (Taberlet *et al.* 1991; primers c and d). We did not analyse the matK barcode because its amplification and sequencing were problematic for some steppic plants (see Heise *et al.* 2015). We did not use primers developed to amplify short barcodes (minibarcodes; e.g. Hofreiter *et al.* 2000 for rbcL and Taberlet *et al.* 1991, 2007 for trnL), as these short markers do not have sufficient discriminatory power and rarely allow for species-level identification (see also Little 2014). As the purpose of this research was to identify host plants to the lowest possible taxonomic level (preferably to the species level), we decided to use standard primers amplifying

Table 1 Efficiency of host plants amplification and sequencing for examined steppic beetle species (ordered according to systematics). Abbreviations: M – monophagous, O – oligophagous, P – polyphagous, U – unknown diet, number of specimens (no. sp.), PCR and sequencing success (√- PCR and sequencing successful, X – unsuccessful, U – sequences unreadable, P – sequences of poor quality, L – lack of BLAST hits) and sequencing technique used for amplification of host plants DNA from the beetles (S – Sanger and I – Illumina)

Species	Phagism	No sp.	PCR & sequencing			Seq. tech.
			COI	rbcL	trnL	
CHRYSOMELOIDEA						
CHRYSOMELIDAE						
Criocerinae						
Crioceris quatuordecimpunctata (Scopoli, 1763)	M	2	√	√	√	S
Crioceris quinquepunctata (Scopoli, 1763)	M	2	√	√	√	S
Cassidinae						
Cassida lineola Creutzer, 1799	M	1	√	√	√	S
Cassida margaritacea Schaller, 1783	O	1	X	X	X	
Cassida panzeri Weise, 1907	M	1	√	L	L	S
Cassida pannonica Suffrian, 1844	M	1	√	√	√	S
Hypocassida subferruginea (Schrank, 1776)	M	1	√	L	L	S
Chrysomelinae						
Chrysolina cerealis (Linnaeus, 1767)	O	1	√	√	√	S
Chrysolina sanguinolenta (Linnaeus, 1758)	M	1	√	√	√	S
Entomoscelis adonidis (Pallas, 1771)	O	1	X	X	X	
Gonioctena fornicata (Brüggemann, 1783)	P	10	√	√	√	I
Gonioctena olivacea (Forster, 1771)	O	1	√	√	√	S
Galerucinae						
Galeruca pomonae (Scopoli, 1763)	U	2	√	L	L	S
Galeruca tanaceti (Linnaeus, 1758)	U	1	√	U	U	S
Calomicrus circumfusus (Marsham, 1802)	O	2	√	√	√	S
Luperus xanthopoda (Schrank, 1781)	O	2	√	√	√	S
Alticinae						
Podagrica fuscicornis (Linnaeus, 1767)	M	1	X	X	X	
Aphthona beckeri Jakobson, 1896	M	1	√	√	√	S
Aphthona cyparissiae (Koch, 1803)	M	2	X	X	X	
Aphthona czwalinai Weise, 1888	M	1	√	√	√	S
Aphthona euphorbiae (Schrank, 1781)	M	1	√	√	√	S
Aphthona lacertosa Rosenhauer, 1847	M	2	√	√	√	S
Aphthona ovata Foudras, 1861	M	1	√	√	√	S
Aphthona pygmaea (Kutschera, 1861)	M	2	√	√	√	S
Aphthona venustula (Kutschera, 1861)	M	2	√	√	√	S
Dibolia cryptocephala (Koch, 1803)	M	1	√	√	√	S
CURCULIONOIDEA						
ANTHRIBIDAE						
Urodontinae						
Bruchela rufipes (Olivier, 1790)	M	1	√	L	L	S
APIONIDAE						
Apioninae						
Hemitrichapion pavidum (Germar, 1817)	M	2	√	√	√	S
Cyanapion platalea (Germar, 1817)	M	1	√	√	√	S
Mesotrichapion punctirostre (Gyllenhal, 1839)	M	2	√	√	√	S
Pseudoperapion brevirostre (Herbst, 1797)	M	2	√	√	√	S
Pseudoprotapion elegantulum (Germar, 1818)	M	2	√	√	√	S
Pseudoprotapion ergenense (Becker, 1864)	M	2	√	√	√	S
Squamapion elongatum (Germar, 1817)	M	2	√	P	√	S
Exapion elongatulum (Desbrochers, 1891)	M	1	X	X	X	
CURCULIONIDAE						
Entiminae						
Argoptochus quadrisignatus (Bach, 1856)	P	12	√	√	√	I
Phyllobius brevis Gyllenhal, 1834	P	12	√	√	√	I
Centricnemus leucogrammus (Germar, 1824)	P	16	√	√	√	I
Cycloderes pilosulus (Herbst, 1795)	P	1	X	X	X	
Eusomus ovulum Germar, 1824	P	16	√	√	√	I
Omias globulus (Boheman, 1843)	U	1	√	√	√	S
Omias puberulus Boheman, 1834	O	1	√	√	√	S
Otiorhynchus fullo (Schrank, 1781)	P	2	√	U	U	S
Paophilus afflatus (Boheman, 1833)	P	1	√	U	U	S
Parafourcartia squamulata (Herbst, 1795)	P	16	√	√	√	I
Philopedon plagiatum (Schaller, 1783)	O	1	√	√	√	S
Polydrusus confluens Stephens, 1831	P	1	√	√	√	S
Polydrusus inustus Germar, 1824	P	16	√	√	√	I
Sitona humeralis Stephens, 1831	M	1	√	√	√	S
Sitona inops Schoenherr, 1832	M	2	√	P	√	S
Sitona languidus Gyllenhal, 1834	M	2	√	√	√	S
Sitona lateralis Gyllenhal, 1834	O	2	√	√	√	S

Table 1 *Continued*

Species	Phagism	No sp.	PCR & sequencing			Seq. tech.
			COI	rbcL	trnL	
Dibolia schillingii (Letzner, 1847)	M	2	√	√	√	S
Longitarsus exsoletus (Linnaeus, 1758)	O	1	√	P	P	S
Longitarsus quadriguttatus (Pontoppidan, 1763)	O	1	X	X	X	
Longitarsus tabidus (Fabricius, 1775)	M	1	√	√	√	S
Neocrepidodera ferruginea (Scopoli, 1763)	O	1	√	√	√	S
Phyllotreta nodicornis (Marsham, 1802)	M	1	√	L	L	S
Podagrica fuscicornis (Linnaeus, 1767)	O	1	√	√	√	S
Psylliodes cucullata (Illiger, 1807)	M	1	√	P	√	S
Sphaeroderma testaceum (Fabricius, 1775)	O	1	√	√	√	
Cryptocephalinae						
Cheilotoma musciformis (Goeze, 1777)	O	12	√	√	√	I
Coptocephala unifasciata (Scopoli, 1763)	P	1	√	√	√	S
Labidostomis humeralis (Schneider, 1792)	M	1	X	X	X	
Labidostomis longimana (Linnaeus, 1760)	P	12	X	√	√	I
Lachnaia sexpunctata (Scopoli, 1763)	O	1	X	X	X	
Smaragdina affinis (Illiger, 1794)	P	10	√	√	√	I
Smaragdina aurita (Linnaeus, 1767)	O	1	√	U	√	S
Cryptocephalus bameuli Duhaldeborde, 1999	P	16	√	U	√	I
Cryptocephalus bilineatus (Linnaeus, 1767)	P	2	√	√	√	S
Cryptocephalus chrysopus Gmelin, 1790	O	2	√	√	√	S
Cryptocephalus flavipes Fabricius, 1781	P	2	√	√	√	S
Cryptocephalus fulvus (Goeze, 1777)	U	2	√	L	√	S
Cryptocephalus pygmaeus Fabricius, 1792	P	10	√	√	√	I
Cryptocephalus quadriguttatus Richter, 1820	U	1	√	U	U	S
Cryptocephalus violaceus Laicharting, 1781	U	14	√	√	√	I
Cryptocephalus virens Suffrian, 1847	O	2	√	√	√	S
Cryptocephalus vittatus Fabricius, 1775	O	2	√	√	√	S
Pachybrachis fimbriolatus (Suffrian, 1848)	P	12	√	√	√	I
Pachybrachis hippophaes (Suffrian, 1848)	O	1	√	L	L	S
Pachybrachis tesselatus (Olivier, 1791)	O	1	√	L	L	S
Eumolpinae						
Chrysochus asclepiadeus (Pallas, 1773)	M	1	√	P	√	S

Species	Phagism	No sp.	PCR & sequencing			Seq. tech.
			COI	rbcL	trnL	
Sitona longulus Gyllenhal, 1834	M	1	√	√	√	S
Sitona striatellus Gyllenhal, 1834	P	12	√	√	√	I
Sitona waterhousei Walton, 1846	O	2	√	√	√	S
Strophosoma faber (Herbst, 1784)	P	1	√	U	U	S
Lixinae						
Larinus obtusus Gyllenhal, 1836	M	3	√	√	√	S
Larinus planus (Fabricius, 1792)	O	1	√	U	U	S
Larinus sturnus (Schaller, 1783)	O	2	√	√	√	S
Larinus turbinatus Gyllenhal, 1836	O	2	√	√	√	S
Hyperinae						
Hypera fuscocinerea (Marsham, 1802)	O	2	√	√	√	S
Curculioninae						
Cionus clairvillei Boheman, 1838	M	2	√	√	√	S
Mecinus pascuorum (Gyllenhal, 1813)	M	1	√	√	√	S
Rhinusa tetra (Fabricius, 1792)	M	3	√	P	√	S
Cleopomiarus distinctus (Boheman, 1845)	M	1	√	√	√	S
Cleopomiarus graminis (Gyllenhal, 1813)	M	2	√	P	√	S
Sibinia subelliptica (Desbrochers, 1873)	M	2	√	√	√	S
Sibinia tibialis (Gyllenhal, 1836)	O	1	√	√	√	S
Sibinia vittata Germar, 1824	M	1				
Tychius aureolus Kiesenwetter, 1851	O	2	√	X	X	
Tychius crassirostris Kirsch, 1871	O	1	√	√	√	S
Tychius schneideri (Herbst, 1795)	M	2	√	√	√	S
Tychius sharpi Tournier, 1873	M	2	√	√	√	S
Tychius medicaginis Brisout, 1862	M	1	√	X	X	
Smicronyx jungermanniae (Reich, 1797)	M	1	√	L	L	S
Pseudorchestes ermischi (Dieckmann, 1958)	M	1	√	√	√	S
Ceutorhynchinae						
Mogulones geographicus (Goeze, 1777)	M	1	X	X	X	
Mogulones javetii (Gerhardt, 1867)	M	1	X	X	X	
Phrydiuchus tau Warner, 1969	M	1	√	√	√	S
Stenocarus ruficornis (Stephens, 1831)	M	1	√	L	L	S
Thamiocolus signatus (Gyllenhal, 1837)	M	1	√	√	√	S
Trichosirocalus barnevillei (Grenier, 1866)	O	1	√	√	√	S
Trichosirocalus troglodytes (Fabricius, 1787)	M	2	√	√	√	S
Zacladus geranii (Paykull, 1800)	M	1	√	L	L	S

longer parts of selected barcodes, or approximately 350–640 bp of trnL intron and 650–680 bp of the rbcL gene. This could potentially lead to an absence of PCR products for some samples (Kajtoch & Mazur 2015), but we reduced the risk of this by using freshly collected and immediately preserved specimens and by using two barcodes.

All PCR products were visualized on an agarose gel, and if more than one band was observed, bands were extracted from the gel using NucleoSpin Gel and PCR Clean-up. PCR products were purified using the Exo-ProStar kit (GE Chemicals). Purified DNA products were then Sanger sequenced using forward primers and a BIGDYE TERMINATOR v.3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and run on an ABI 3100 Automated Capillary DNA Sequencer. In cases of unreadable sequences, the sequencing procedure was repeated under modified PCR conditions with the use of reverse primers.

For seven species of weevils and eight species of leaf beetles (mostly polyphagous, see Table 1), another method of host plant identification was used. Barcodes of rbcL and trnL were amplified separately for each individual to avoid problems and errors caused by an unequal concentration of plant DNA in isolates from weevil bodies. Between 10 and 16 specimens of each species were used (see Table 1 for details). All amplicons (small volumes of both rbcL and trnL) were first checked on agarose gel and then pooled approximately equimolarly (separately for each species; all rbcL PCRs were pooled separately from trnL PCRs) and purified using the NucleoSpin DNA extraction kit. Each batch of PCRs included blank samples (with all reagents but without DNA templates) to test for possible contamination. None of these negative controls resulted in a PCR product. The barcoded libraries were prepared using NEBNext DNA library prep without the DNA fragmentation step, that is adaptors were ligated to the amplicon ends. The libraries were sequenced as part of a MiSeq paired-end 2 × 300 bp run, which allowed for sequencing of the full, or almost full, length of trnL and most of the length of rbcL barcodes.

Data analysis

Host plant identification. Sanger sequences. Sanger sequences were checked visually using BIOEDIT v.7.0.5.2 (Hall 1999). Only good-quality sequences longer than 350 bp (trnL, mostly longer than 500 bp) or 600 bp (rbcL) were used for further analysis. Two approaches were used for host plant identification in Sanger sequenced samples. First, Sanger sequences of both barcodes obtained from beetle guts were compared with the available databases of xerothermic plant barcodes (Heise *et al.* 2015) using MEGABLAST (Altschul *et al.* 1990). Only hits with at least 99% identity, E-value $<10^{-200}$ and >95% query coverage were retained. These thresholds were set somewhat arbitrarily to maximize the stringency of identification of the host plant species. Query coverage of at least 95% was required to exclude, for example, chimeric sequences that may have been generated during PCR. An identity of at least 99% was chosen to allow for sequencing errors and intraspecific genetic variation. An alternative approach for host plant identification from Sanger sequences was based on phylogenetic analysis (Mitter & Brooks 1983; Mitter *et al.* 1991; Miller & Wenzel 1995). To visualize plants featured in the diets of the two beetle families in the context of the species present in the previously compiled database of steppic plants, we constructed a phylogenetic tree using sequences obtained from the beetles and from the database. We selected the rbcL barcode for phylogenetic host plant identification as this gene could be easily and reliably aligned, contrary to the indel-rich trnL intron. All rbcL sequences generated from the beetles were added to rbcL sequences from the barcode database, and the data set was aligned using MAFFT v.7 (Katoh & Standley 2013). The Akaike information criterion (AIC) in MRMODELTEST 2.3 (Nylander 2004) in conjunction with PAUP* (Swofford 2002) was used to determine the best-fitting nucleotide substitution model. Next, we used PHYML 3.0 (Guindon *et al.* 2010) to reconstruct a maximum-likelihood phylogenetic tree. PHYML was run with an appropriate substitution model, and node support was assessed with the bootstrap technique using 1000 pseudo-replicates. The tree was visualized and edited with FIGTREE v1.3.1 (Rambaut 2009). Sequences generated directly from plants, weevils and leaf beetles were marked with distinct colours.

Host plant identification. Illumina sequences. We used the following approach to analyse the Illumina sequences obtained from 15 beetle species. Both paired reads were joined end to end, and only joined reads of length larger than 300 bp were used in further analyses. End-to-end joining was necessary because rbcL and in many plant species also trnL amplicons are longer than 600 bp, so the 300-bp reads from both amplicon ends did not overlap. This procedure may have resulted in duplications in the middle of the joined sequence if the paired-end reads overlapped (for trnL amplicons shorter than 600 bp which occur in some plant species). Duplicated fragments in the middle of the reads should not significantly affect blast sensitivity as it uses a local alignment approach. Identification of the plant was performed by a comparison of the sequencing reads with sequences in the database of plant barcodes. Plant identification was performed by comparing sequencing

reads with sequences in the database of plant barcodes. For each ≥300-bp read, an ungapped MEGABLAST search with the cut-off E-value of 10^{-150} was performed with the maximum of 10 hits retained. Only reads with the best hits showing at least 98% identity to at least one plant species in the database were retained. This threshold was estimated on the basis of divergence analyses made for all available steppic plants in a previous study (Heise *et al.* 2015). Moreover, 98% identity was used in other studies that performed host plant identification using plant barcodes and next-generation sequencing technologies (e.g. Soininen *et al.* 2009; Valentini *et al.* 2009b; Hajibabaei *et al.* 2011; Heise *et al.* 2015). A read was considered to have a unique match if only a single hit was reported or if the bit score of the second best hit was no better than 0.95× the bit score of the best hit; if multiple high-scoring pairs [hsp] occurred for a given query, these were combined. Host plant species were identified only on the basis of these reads. This procedure has recently been successfully tested on a polyphagous beetle (see Heise *et al.* 2015).

We also validated this method for the identification of known plant species in a mixed sample. Amplicons of both barcodes obtained for the eight selected plant species (one from each family: *Eryngium planum*, *Inula ensifolia*, *Onobrychis viciifolia*, *Adonis vernalis*, *Salvia pratensis*, *Rosa canina*, *Arenaria serpyllifolia* and *Elymus repens*) were pooled, Illumina sequenced and analysed as described above.

Beetle–host plant analysis. COI sequences generated for beetles were aligned using MAFFT v.7, and the best-fitting nucleotide substitution model was determined using AIC in MRMODELTEST 2.3 in conjunction with PAUP*. Phylogenetic trees using the maximum-likelihood approach were constructed separately in PHYML 3.0 for weevils and leaf beetles. Five beetle species were used as out-groups (sequences downloaded from GenBank): *Nyctoporis carinata* (Tenebrionidae; EU037102), *Coraebus elatus* (Buprestidae; JQ303296), *Melanotus communis* (Elateridae; EF424474), *Arachnodes emmae* (Scarabaeidae; GQ342139) and *Platycerus virescens* (Lucanidae; AB609585). These species were randomly selected among representatives of distant (in respect to weevils and leaf beetles) beetle families. The COI trees showing relationships of beetle species were then used for the preparation of networks visualizing all interactions identified between the beetles and their host plants (combining information across barcodes and sequencing technologies). Due to a large number of such interactions, we decided to visualize these networks in a simplified way, connecting the beetle species to their host plants at the family level (details about the host plant species are presented in additional tables). Each beetle species for which a host plant was identified based on plant DNA barcoding using a comparison of data from the barcodes to information from the literature (based on information collected from Burakowski *et al.* 1990a,b, 1991, 1992, 1995, 1997, and other works cited above) was analysed to evaluate the congruence between current and older knowledge about beetle feeding preferences.

Next, data about the host plants of the steppic beetles, which were combined from barcodes and sequencing technologies, were used for a general analysis of the feeding preferences of beetles. Steppic plant species were assigned as host plants for certain beetle species on the basis of barcode identification. The matrix was then used to calculate the number of beetle species [separately for weevils polyphagous and mono/oligophagous (monophagous and oligophagous) and simultaneously for leaf beetles] feeding on a particular plant species. This analysis was performed on all beetle species that had at least one identified host plant. To check the correlation between the number of beetle species feeding on a particular plant species, we used Pearson's correlation (R). Additionally, cluster analysis was implemented to visualize relative clustering of four defined groups of beetles in the area of feeding on particular plant species. Differences in the composition of plants consumed by the above four groups of beetles were also tested with use of analysis of variance (ANOVA). All statistical analyses were performed using STATISTICA 10.0 (Statsoft). Finally, using ESTIMATES (Colwell 2013), we calculated the Bray–Curtis dissimilarity index (BC) (Bray & Curtis 1957) between (i) polyphagous weevils and leaf beetles, (ii) mono/oligophagous weevils and leaf beetles, (iii) polyphagous and mono/oligophagous weevils, and (iv) polyphagous and mono/oligophagous leaf beetles.

Results

Sampling efficiency

Despite the rarity of many steppic beetle species, we managed to collect 55 species of leaf beetles (i.e. 65% of species associated with steppic grasslands in Poland and central Europe; Borowiec *et al.* 2011; Schmitt & Rönn 2011) and 59 species of weevils (52% of species from central Europe; Mazur 2001; Wanat & Mokrzycki 2005; see Table 1). Species that could not be collected included extremely rare beetles often restricted to single localities (e.g. the weevil *Donus nidensis*, known only in one steppic patch in southern Poland and another in western Ukraine; *Timarcha rugulosa*, a very rare species known only from a few localities). We intentionally omitted some of these species from our study,

regardless of their conservation status, as the collection of even single individuals could be detrimental for their local populations (Kajtoch *et al.* 2014).

General diet characterizations of steppic beetle species

PCR failure rates were 14.9% for leaf beetles and 15.7% for weevils. For the majority of the beetles (66% of leaf beetles and 67% of weevils), Sanger sequencing allowed for the identification of the host plants (see Table S1 and Appendix S1, Supporting information for details). Similarly, the phylogenetic approach based on rbcL sequences allowed for host plant identification for 62% of leaf beetle species and 65% of weevil species (see Fig. S1, Supporting information). All eight species of plants preselected for the validation procedure were identified in Illumina generated sequences blasted against the reference barcode database (see Table S2 and Appendix S2, Supporting information for details).

The efficiency of Illumina sequencing on plant DNA isolated from beetles and the results of host plant identification (number of hits to particular host plants identified for examined beetles and relative frequencies of identified host plants in groups of sequences generated for the beetle species) are presented in Appendix S3 (Supporting information). The following numbers of host plants per species were identified by Illumina sequencing: based on trnL, weevils: 7.4 ± (SD) 1.02 (range 3–12); leaf beetles: 6.1 ± 1.83 (2–18); based on rbcL, weevils: 6.7 ± 0.64 (5–10); and leaf beetles: 7.8 ± 1.97 (3–18). The most polyphagous weevils were as follows: *Centricnemus leucogrammus* (16 host plants), *Argoptochus quadrisignatus* (12), *Polydrusus inustus* (11), and *Eusomus ovulum* (10). The most polyphagous leaf beetles were *Cryptocephalus bameuli* (27), *Cryptocephalus pygmaeus* (18), and *Gonioctena fornicata* (15).

Barcoding vs. the direct observation of feeding beetles

For species whose host plants were identified unambiguously, including the vast majority of monophagous beetles (94% of leaf beetles and 100% of weevils), the barcoding approach identified the same host plant that was previously reported on the basis of direct observations (Table S1, Supporting information). Similarly, an overwhelming majority of oligophagous species (91% of leaf beetles and 90% of weevils) were found to feed on plants belonging to the same plant genus or to one of the species belonging to the plant family known as hosts for the particular beetle (Table S1, Supporting information). Moreover, almost all species classified as polyphagous by direct observations were confirmed to feed on multiple hosts, with the single exception of *Cryptocephalus violaceus,* which is apparently associated with only two plant genera (see Table S2, Supporting information).

Differences in diet composition between beetle families (weevils vs. leaf beetles)

Networks of interactions between weevils and their host plants and leaf beetles and their host plants were found to be complex. In total, we identified 224 beetle–host plant interactions (117 for leaf beetles and 107 for weevils; see Figs 1 and 2). However, when we exclude polyphagous taxa, the number of interactions decreases substantially to only 65 (31 for leaf beetles and 34 for weevils; see Table S1, Supporting information, Figs 1 and 2).

The polyphagous species most commonly ate the following host plants: *Onobrychis viciifolia* (the host plant of 6–7% of the studied beetles), *Hypericum perforatum* (the host plant of 6% of leaf beetles), *Lotus corniculatus* (4.3% of leaf beetles), *Prunus spinosa* (4.3% of weevils and 3.4% of leaf beetles), *Crataegus monogyna* (3.4% of both weevils and leaf beetles), *Salvia pratensis* (2.6% of both weevils and leaf beetles), *Filipendula vulgaris* (2.6% of weevils and 3.4% of leaf beetles) and *Sarothamnus scoparius* (3.4% of weevils; see Fig. 2 for details). At the family level, the most commonly eaten host plants were Fabaceae (14.5% of weevils and 23.9% of leaf beetles), Rosaceae (20.5% and 17.1%, respectively), Asteraceae (5.1% and 8.5%) and Lamiaceae (both 6.0%; see Figs 1 and 2). There was a significant correlation between the number of polyphagous leaf beetle and weevil species feeding on particular plant species ($R = 0.704$, $P < 0.001$). The BC dissimilarity index between these two groups was 0.39.

A different pattern was observed when polyphagous species were omitted. The plants most often consumed by monophagous and oligophagous beetles were as follows: *Euphorbia cyparissias* (16.1% of leaf beetles), *Cirsium pannonicum, Linum flavum, Genista tinctoria, Asparagus officinalis* (6.5% of leaf beetles each), *Medicago varia* (8.9% of weevils), *Centaurea scabiosa, Campanula glomerata, Salvia pratensis, Plantago lanceolata, Verbascum lychnitis, Coronilla varia, Lathyrus tuberosus, Onobrychis viciifolia* and *Trifolium arvense* (5.9% of weevils each; see Fig. 2). Differences between leaf beetles and weevils were also observed at the host plant family level: Asteraceae (16.1% of leaf beetles and 14.7% of weevils), Hypericaceae (16.1% of leaf beetles), Rosaceae (9.7% of leaf beetles) and Lamiaceae (8.8% of weevils; details in Figs 1 and 2). There was no significant correlation between numbers of mono/oligophagous leaf beetle and weevil species feeding on particular plant species ($r = 0.107$, $P = 0.380$). The BC dissimilarity index between these two groups was 0.75.

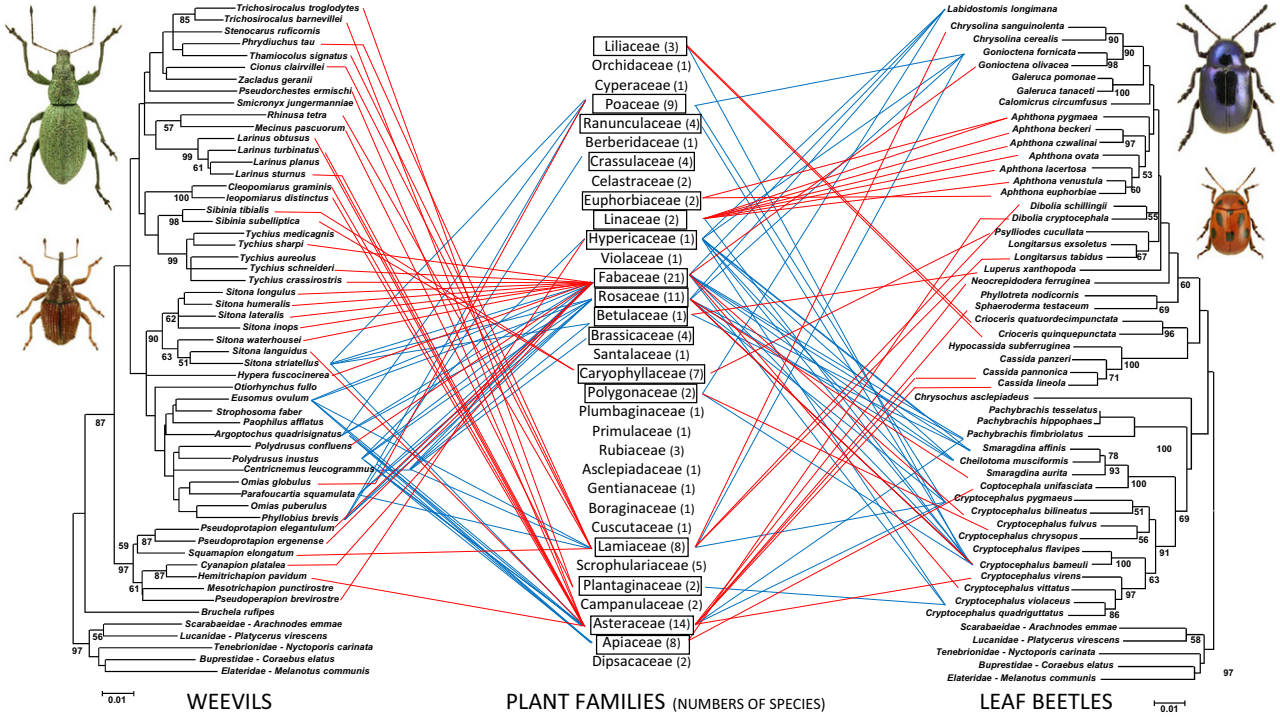

Fig. 1 Networks of interactions identified among herbivorous beetles inhabiting steppic grasslands in central Europe and their host plants. Weevils and leaf beetles are presented in maximum-likelihood phylogenetic trees reconstructed from sequences of the cytochrome oxidase I mitochondrial gene (only bootstraps with a value of >50% are presented). Numbers in brackets presented along with names of all steppic plant families for which barcodes were available (Heise *et al.* 2015) express the numbers of plant species in the families. Plant families eaten by any of beetles are marked in frames. Interactions for beetles with Sanger sequencing data (mostly monophagous or oligophagous taxa) are marked in red, and interactions for beetles with Illumina sequencing data (mostly polyphagous taxa) are marked in blue. *Labidostomis longimana* leaf beetles are presented outside the COI as it was not possible to generate a homologous sequence for this species. Images of weevils: *Eusomus ovulum* (top) and *Trichosirocalus troglodytes* (bottom) and leaf beetles: *Chrysochus asclepiadeus* (top) and *Gonioctena fornicata* (bottom) [photographs are from ICONOGRAPHIA COLEOPTERORUM POLONIAE (© Copyright by Prof. Lech Borowiec, Wrocław 2007–2014, Department of Biodiversity and Evolutionary Taxonomy, University of Wroclaw, Poland)].

Moreover, when comparing polyphagous and mono/oligophagous leaf beetles, no correlation was found ($R = 0.204$, $P = 0.092$), as both of these groups were highly dissimilar (BC index = 0.79). The same was observed for polyphagous and mono/oligophagous weevils ($R = 0.152$, $P = 0.201$; BC index = 0.74). All defined groups of beetles differed significantly in food selection (ANOVA = 24.78, $P < 0001$; see also Fig. S2, Supporting information).

Differences in diet composition among congeneric species

When analysing the feeding preferences of congeneric beetle species, three groups could be identified. The first contains members of the genera that feed on the same host plants: *Crioceris* (feeding exclusively on *Asparagus*), *Cleopomiarus* (with the *Campanula* host plant) and *Pseudoprotapion* (monophages of *Onobrychis*). The second group includes beetle genera that feed on two or more genera of plants that are often phylogenetically related, for example *Tychius* (feeding on *Trifolium* or *Melilotus* – Fabaceae), *Sibinia* (*Silene* and *Dianthus*, both from Caryophyllaceae), *Larinus* (*Carlina*, *Centaurea and Cirsium* – all from Asteraceae), *Apthona* (feeding mostly on *Euphorbia*, but some on *Linum*) and *Sitona* (feeding mostly on Fabaceae). Genera belonging to the third group include beetles feeding on different, unrelated plants: *Cryptocephalus* (with some polyphagous species), *Polydrusus* (generally polyphagous), *Cassida* and *Trichosirocalus*.

Some genera of steppic weevils and leaf beetles contain both polyphagous and monophagous species (e.g. *Cryptocephalus*, *Cassida*, *Chrysolina*, *Pachybrachis*, *Sibinia*, *Sitona* and *Trichosirocalus*). In other genera, all of the examined steppic species are either monophagous/oligophagous (*Aphthona*, *Dibolia*, *Gonioctena*, *Longitarsus*, *Hemitrichapion*, *Miarus*, *Pseudoprotapion* and *Tychius*) or polyphagous (*Galeruca*, *Labidostomis*, *Smaragdina*, *Larinus* and *Polydrusus*). Overall, a transition between mono/oligophagy and polyphagy was observed in 30% of genera

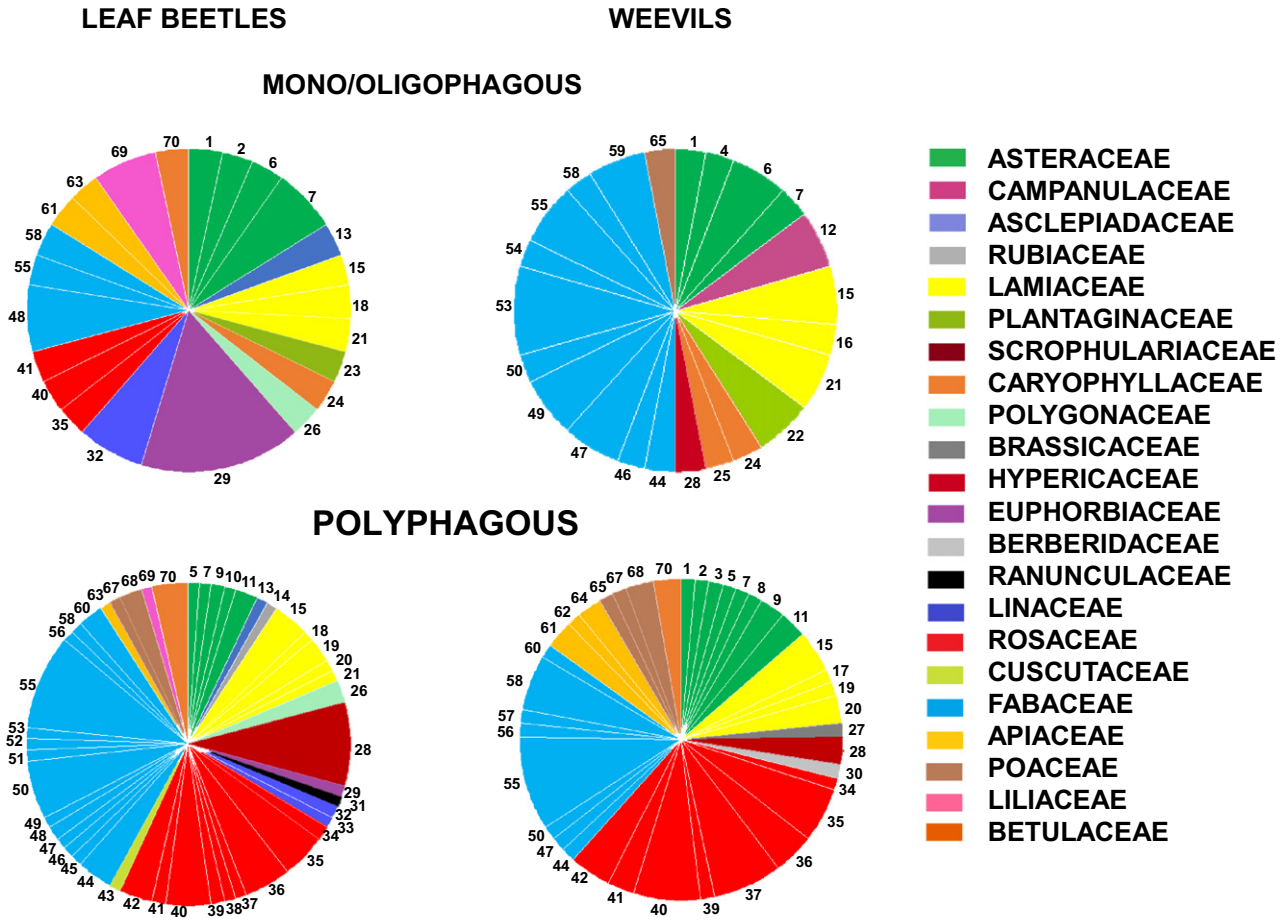

Fig. 2 Host plant composition of steppic beetles (leaf beetles and weevils) showed for all examined beetles (top drawings) and for only species with single host plants identified (bottom drawings). Colours correspond to particular plant families; numbers indicate plant species as follows: *1- Achillea millefolium, 2 - Artemisia campestris, 3 - Carlina acaulis, 4 - Carlina onopordifolia, 5 - Centaurea stoebe, 6 - Centaurea scabiosa, 7 - Cirsium pannonicum, 8 - Hieracium pilosella, 9 - Inula ensifolia, 10 - Picris hieracioides, 11 - Chrysanthemum corymbosum, 12 - Campanula glomerata, 13 - Vincetoxicum hirundinaria, 14 - Galium mollugo, 15 - Salvia pratensis, 16 - Stachys recta, 17 - Teucrium chamaedrys, 18 - Thymus pannonicus, 19 - Thymus pulegioides, 20 - Clinopodium vulgare, 21 - Verbascum lychnitis, 22 - Plantago lanceolata, 23 - Linaria vulgaris, 24 - Dianthus carthusianorum, 25 - Silene nutans, 26 - Rumex acetosella, 27 - Sisymbrium loeselii, 28 - Hypericum perforatum, 29 - Euphorbia cyparissias, 30 - Berberis vulgaris, 31 - Ranunculus acris, 32 - Linum flavum, 33 - Linum hirsutum, 34 - Agrimonia eupatoria, 35 - Crataegus monogyna, 36 - Filipendula vulgaris, 37 - Fragaria viridis, 38 - Potentilla alba, 39 - Potentilla argentea, 40 - Prunus spinosa, 41 - Rosa canina, 42 - Sanguisorba minor, 43 - Cuscuta epithymum, 44 - Anthyllis vulneraria, 45 - Astragalus arenarius, 46 - Astragalus danicus, 47 - Coronilla varia, 48 - Genista tinctoria, 49 - Lathyrus tuberosus, 50 - Lotus corniculatus, 51 - Medicago falcata, 52 - Medicago lupulina, 53 - Medicago varia, 54 - Melilotus officinalis, 55 - Onobrychis viciifolia, 56 - Ononis spinosa, 57 - Oxytropis pilosa, 58 - Sarothamnus scoparius, 59 - Trifolium arvense, 60 - Vicia tenuifolia, 61 - Eryngium planum, 62 - Seseli libanotis, 63 - Peucedanum cervaria, 64 - Pimpinella saxifraga, 65 - Elymus repens, 66 - Festuca rupicola, 67 - Koeleria macrantha, 68 - Stipa Joannis, 69 - Asparagus officinalis, 70 - Corylus avellana.*

(4 of 12 leaf beetles and 9 of 12 weevils, only considering genera represented by at least two species in our study).

Discussion

Here, we present the first analysis of ecological associations between herbivorous beetles and their host plants from steppic grasslands, a highly threatened environment in central Europe. Comprehensive analyses using two DNA barcodes and two sequencing technologies have significantly expanded knowledge about feeding preferences for this ecological guild of beetles.

Accuracy and reliability of direct observations of feeding

For monophagous and most oligophagous species, host plant identification base.d on DNA barcoding generally

agreed with previously published information about their feeding preferences. This finding confirms that traditional studies, mostly direct observations of beetles in the field (Freude *et al.* 1966, 1981; Dieckmann 1980; Burakowski *et al.* 1990a,b, 1991, 1992, 1995, 1997), correctly identified host plants to these beetle species. However, we also found some discrepancies in host plant identification based on observations and barcodes. One of the most interesting findings is the identification of multiple host plants for the leaf beetle *Cheilotoma musciformis*, which, based on observations, should feed only on *Onobrychis*, *Anthyllis* and *Rumex* (Szymczakowski 1960; Gruev & Tomov 1984; Warchałowski 1991). Previous studies on limited samples indicate that this species is oligophagous and feed exclusively on Fabaceae (*Onobrychis*, *Lotus* and *Oxytropis*; Kajtoch *et al.* 2013; Heise *et al.* 2015), whereas the current study extends the list of its host plants to include some Rosaceae (e.g. *Prunus* and *Crataegus*) and *Hypericum*.

Novel findings on diet preferences of steppic beetles

One interesting result was the identification of host plants for species with previously unknown diets. Examples are the weevil *Omias globulus*, which feeds on *Elymus repens*, and leaf beetle *Cryptocephalus violaceus*, which feeds on *Onobrychis* and *Hypericum*. Illumina sequencing of barcodes generated from several randomly picked individuals showed that some presumably polyphagous species are rather oligophagous (e.g. the weevil *Sitona striatellus* and leaf beetles *Labidostomis longimana* and *Smaragdina affinis*) and feed on two or only a few plants. Other species are polyphagous, but with a diet restricted to some plant families (like weevils: *Polydrus inustus* – Rosaceae, *Eusomus ovulum* – Rosaceae and Fabaceae, leaf beetles *Gonioctena fornicata* – Rosaceae and Fabaceae; see also Table S2, Supporting information).

Does diet composition differ between weevils and leaf beetles?

Weevils and leaf beetles constitute more than half of all beetle species associated with steppic grasslands (Mazur 2001; Wanat & Mokrzycki 2005; Wąsowska 2006; Borowiec *et al.* 2011; Mazur & Kubisz 2013). They are phylogenetically distant but closely linked ecologically and show similar feeding habits (mainly leaf-eaters as imago). Data collected for dozens of steppic species from both families gave us a unique opportunity to comprehensively compare these two groups. Two opposite patterns are observed for polyphagous and mono/oligophagous species. In polyphagous species, weevils and leaf beetles feed on nearly the same plant species. This result simply confirms that polyphagous steppic beetles are feeding generalists (Bernays & Minkenberg 1997). The polyphagous species could simply follow the abundance and constancy of plants in the environment. Another explanation for this lack of difference in the diets of polyphagous weevils and leaf beetles is that all of these species feed on plants which have less effective chemical defences, which implies that generalists are less adapted to repellents than specialists, who are probably specifically adapted. Polyphagous species were responsible for approximately two-thirds of the links between host plants and beetles. When only mono/oligophagous beetles were analysed, significant differences in host plant composition were detected between weevils and leaf beetles. Only members of Asteraceae and Lamiaceae are similarly important as host plants for both groups of beetles, whereas other plant families were more frequent in the diet of either weevils or leaf beetles. This suggests some dietary niche displacement between these two groups of beetles caused by host plant specificity. Such specificity could have resulted from competition for available food resources during the evolution of both groups. It accelerated when seed plant radiation began, as host plant selection is currently considered to be one of the major forces of beetle speciation and insect speciation in general (Thorsteinson 1960; Ward *et al.* 2003; Grimaldi & Engel 2005). Another probable explanation is the avoidance of some host plants possessing efficient chemical defences (Schultz 1988; Hartley & Jones 1997; Aniszewski 2007). However, due to the different physiology of particular species, leaf beetles and weevils could be adapted to feed on plants with different repellents. Both processes are not exclusive and have often led to co-evolution between herbivores and their host plants. This has been observed particularly in beetles (e.g. Petitpierre & Segarra 1985; Metcalf 1986; Anderson 1993; Farrell 1998; Oberprieler *et al.* 2007; Lawrence *et al.* 2011).

Intrageneric competition for food plant resources

This dietary displacement could be associated with beetle phylogeny, and, if so, it should be observed mainly between closely related species (Petitpierre & Segarra 1985; Metcalf 1986; Anderson 1993; Farrell 1998; Oberprieler *et al.* 2007; Lawrence *et al.* 2011). However, only in some genera that were represented by multiple species in the study did we find that the diets of related species are substantially different. Species belonging to the same genera feed on different plants, usually from other genera or families (e.g. *Cassida* and *Trichosirocalus*, and to a lesser extent also *Apthona*, *Larinus*, *Sibinia*, *Sitona* and *Tychius*,). This can also be explained as either

food competition avoidance or adaptation to hosts with different chemical defences. In some genera, species feed on different, unrelated plants and all or some of these beetles are polyphagous (e.g. *Cryptocephalus* and *Polydrusus*). In these genera, the feeding preferences of particular species probably evolved as a way to feed on multiple host plants, which could also reduce congeneric competition (e.g. for more nutritious plants) or, again, could be a result of adaptation to different insect repellents present in plants. Moreover, we found that in the evolutionary history of approximately one-third of the studied beetle genera, some shifts between monophagy, oligophagy and poliphagy happened; similar shifts were reported for some other beetles (e.g. *Oreina*, Dobler *et al.* 1996). However, these transitional events could pre-date the formation of steppic assemblages. On the other hand, only some beetle genera were found to feed exclusively on the same host plants (e.g. *Crioceris*, *Cleopomiarus* and *Pseudoprotapion*). Species from these genera apparently maintained general feeding preferences from common ancestors, as was shown for some other beetle genera (e.g. *Phyllobrotica,* Farrell & Mitter 1990; *Ophraella,* Futuyma *et al.* 1995; *Anthonomus grandis* species group, Jones 2001).

Limitations of host plant barcoding

We are aware of the limitations of this study, including limited sampling for some species and some technical constrains (see Appendix S4, Supporting information for details). Limited sampling could have resulted in the underestimation of host plant diversity in the diet of oligophagous species, but this should not affect most of the results, especially those on higher grouping levels such as the analyses of weevils vs. leaf beetles and mono/oligophagous vs. polyphagous species. PCR failure, sequencing errors or problems with species assignment to the reference barcode database were also reported in similar studies (see Jurado-Rivera *et al.* 2009; Pinzón-Navarro *et al.* 2010; Kishimoto *et al.* 2013), and we tried to minimize biases caused by these technical constraints. In our opinion, the presented results adequately reflect the trophic relations between steppic beetles and their host plants.

It is also important to emphasize that data in Table S2 (Supporting information) should not be considered as quantitative, that is corresponding to the actual contribution of various plant species to the diet of particular beetle species. Multiple factors, such as variation among plant species in the rates of digestion, the efficiency of DNA extraction and the process of PCR amplification, most likely introduce considerable bias, and thus, the data can be regarded as semiquantitative at best.

Conservation implications

Apart from the ecological implications of this study, the identification of host plants for beetles could be crucial from a conservation point of view. Steppic grasslands are presently highly fragmented. Patches of this habitat are usually isolated from one another, and the gene flow between populations is limited (see Kajtoch *et al.* 2014).

Only some steppic patches in protected areas (usually very small) remain in good condition (Eriksson *et al.* 2002; Janišová *et al.* 2011; Wesche *et al.* 2012). Consequently, both populations of steppic plants and animals are highly threatened. For the effective conservation of steppic populations and management of steppic habitats, an extensive knowledge about local flora and fauna is needed; however, relatively little is known about the ecology of steppic invertebrates. Despite their rarity, steppic beetle species are not protected under local or international (e.g. European Union) laws. Consequently, steppic grasslands are protected mainly as localities that are important for other taxa, such as orchids (Natura 2000 sites). However, the effective protection of steppic patches should include conservation priorities not only for these 'flagship' plants (which are not found to be hosts for any of the beetles examined in this study), but also for all other steppic organisms (also a common issue for other habitats and species; see Cardoso 2012). The planning of any conservation actions in steppic grasslands needs to be rooted in basic knowledge about the species inhabiting the area, including herbivorous beetles, as it could be crucial for the survival of their populations to sustain certain plants. This concerns mainly mono/oligophagous species, which depend on single or several host plants. Some plants from Fabaceae, Rosaceae, Lamiaceae and Hypericaceae, which are most frequently eaten by beetles, are the most preferred food for domestic mammals and are also utilized by humans (collected in grasslands mostly as herbs or fruits). Knowledge about the host plants of beetles and other steppic species could be even more important if they are re-introduced or translocated to preserve or restore at-risk or locally extinct populations. Such actions would be futile unless the preferred host plants are confirmed in the patches used for beetle settlement or these plants are translocated along with the beetles.

Acknowledgements

This study was funded by the grant of National Science Centre, Poland (UMO-2011/01/B/NZ8/01491, Principal Investigator – Kajtoch Ł.).

We would like to thank K. Dudek for performing library preparation and Illumina sequencing. We thank Brent Emerson and four anonymous reviewers for their helpful comments on

the previous version of this article. This study complies with Laws on Animal Experimentation and Sampling from Natural Populations (none of beetle species used in the study is under protection in Poland and other central European countries where sampling was performed).

Authors declare no conflict of interests.

References

Agrawal AA (2007) Macroevolution of plant defense strategies. *Trends in Ecology & Evolution*, **22**, 103–109.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403–410.

Anderson RS (1993) Weevils and plants: phylogenetic versus ecological mediation of evolution of host plant associations in curculionidae (Curculioninae). *Memoirs of the Entomological Society of Canada*, **165**, 197–232.

Aniszewski T (2007) *Alkaloids – Secrets of Life: Alkaloid Chemistry, Biological Significance, Applications and Ecological Role*. Elsevier, Amsterdam.

Avanesyan A (2014) Plant DNA detection from grasshopper guts: a step-by-step protocol, from tissue preparation to obtaining plant DNA sequences. *Applications in Plant Sciences*, **2**, 1300082.

Barone JA (1998) Host-specificity of folivorous insects in a moist tropical forest. *Journal of Animal Ecology*, **67**, 400–409.

Becerra JX (1997) Insects on plants: macroevolutionary chemical trends in host use. *Science*, **276**, 253–256.

Becerra JX, Venable E (1999) Macroevolution of insect-plant associations: the relevance of host biogeography to host affiliation. *Proceedings of the National Academy of Sciences, USA*, **96**, 12626–12631.

Bernays EA, Chapman RF (1994) *Host-Plant Selection by Phytophagous Insects*. Chapman and Hall, New York, New York.

Bernays EA, Minkenberg OPJM (1997) Insect herbivores: different reasons for being a generalist. *Ecology (Washington DC)*, **78**, 1157–1169.

Binot M, Bless R, Boye P, Gruttke H, Pretscher P (eds.) (1998) Rote liste gefährdeter tiere Deutschlands. *Schriftenreihe für Landschaftspflege und Naturschutz*, **55**, 1–43. (in german).

Borowiec L, Ścibior R, Kubisz D (2011) Critical check-list of the Polish Chrysomeloidea, excluding Cerambycidae (Coleoptera: Phytophaga). *Genus*, **22**, 79–608.

Bray JR, Curtis JT (1957) An ordination of upland forest communities of southern Wisconsin. *Ecological Monographs*, **27**, 325–349.

Burakowski B, Mroczkowski M, Stefanska J (1990a) *Chrzaszcze Coleoptera. Cerambycidae i Bruchidae. Katalog Fauny Polski*. Polish Scientific Publisher, Warsaw, Poland (in polish).

Burakowski B, Mroczkowski M, Stefanska J (1990b) *Chrzaszcze Coleoptera. Stonkowate – Chrysomelidae, p.1. Katalog Fauny Polski*. Polish Scientific Publisher, Warsaw, Poland (in polish).

Burakowski B, Mroczkowski M, Stefanska J (1991) *Chrzaszcze Coleoptera. Stonkowate – Chrysomelidae, p.2. Katalog Fauny Polski*. Polish Scientific Publisher, Warsaw, Poland (in polish).

Burakowski B, Mroczkowski M, Stefanska J (1992) *Chrzaszcze Coleoptera. Ryjkowcowate Prócz Ryjkowców – Curculionoidea Prócz Curculionidae. Katalog Fauny Polski*. Museum and Institute of Zoology Polish Academy of Sciences, Warsaw, Poland (in polish).

Burakowski B, Mroczkowski M, Stefanska J (1995) *Chrzaszcze Coleoptera. Ryjkowce – Curculionidae, p. 2. Katalog Fauny Polski*. Museum and Institute of Zoology Polish Academy of Science, Warsaw, Poland (in polish).

Burakowski B, Mroczkowski M, Stefanska J (1997) *Chrzaszcze Coleoptera. Ryjkowce – Curculionidae, p. 3. Katalog Fauny Polski*. Museum and Institute of Zoology Polish Academy of Science, Warsaw, Poland (in polish).

Cardoso P (2012) Habitats directive species lists: urgent need of revision. *Insect Conservation Diversity*, **5**, 169–174.

Ceynowa M (1968) Zbiorowiska roślinności kserotermicznej nad Dolną Wisłą. *Studia Societatis Scientiarum Torunensis. Sectio D (Botanica)*, **8**, 3–148. (in polish).

Chytrý M (ed.) (2007) *Vegetace Ceské Republiky 1. Travinná a Kerícková Vegetace. Vegetation of the Czech Republic 1. Grassland and Heathland Vegetation*. Academia, Praha, Czech Republic (in czech).

Colwell RK (2013) *Estimate: Statistical Estimation of Species Richness and Shared Species From Samples*. Version 9. User's Guide and application published at: http://purl.oclc.org/estimates.

Dahle B, Sørensen OJ, Wedul EH, Swenson JE, Sandegren F (1998) The diet of brown bears *Ursus arctos* in central Scandinavia: effect of access to free-ranging domestic sheep *Ovis aries*. *Wildlife Biology*, **4**, 147–158.

Dieckmann L (1980) Beiträge zur Insektenfauna der DDR: coleoptera – Curculionidae (Brachycerinae, Otiorhynchina, Brachyderinae). *Beiträge zur Entomologie*, **30**, 145–310.

Dobler S, Mardulyn P, Pasteels JM, Rowell-Rahier M (1996) Host-plant switches and the evolution of chemical defense and life history in the leaf beetle genus Oreina. *Evolution*, **50**, 2373–2386.

Dove H, Mayes RW (1996) Plant wax components: a new approach to estimating intake and diet composition in herbivores. *Journal of Nutrition*, **126**, 13–26.

Dyer LA, Singer MS, Lill JT *et al.* (2007) Host specificity of Lepidoptera in tropicaland temperate forests. *Nature*, **448**, 696–699.

Ehrlich PR, Raven PH (1964) Butterflies and plants: a study in coevolution. *Evolution*, **18**, 586–608.

Eriksson O, Cousins S, Bruun HH (2002) Land-use history and fragmentation of traditionally managed grasslands in Scandinavia. *Journal of Vegetation Science*, **13**, 743–748.

Farkač J, Král D, Škorpík M (eds) (2005) *Cervený Seznam Ohrožených Druhu Ceské Republiky*. Bezobratlí, Agentura ochrany prírody a krajiny CR, Praha, Czech Republic (in czech).

Farrell BD (1998) "Inordinate fondness" explained: why are there so many beetles? *Science*, **281**, 555–559.

Farrell B, Mitter C (1990) Phylogenesis of insect/plant interactions: have Phyllobrotica leaf beetles (Chrysomelidae) and the Lamiales diversified in parallel? *Evolution*, **44**, 1389–1403.

Fay MF, Swensen SM, Chase MW (1997) Taxonomic affinities of *Medusagyne oppositifolia* (Medusagynaceae). *Kew Bulletin*, **52**, 111–120.

Foley WJ, McIlwee A, Lawler I, Aragones L, Woolnough AP, Berding N (1998) Ecological applications of near infrared reflectance spectroscopy – a tool for rapid, cost-effective prediction of the composition of plant and animal tissues and aspects of animal performance. *Oecologia*, **116**, 293–305.

Freude H, Harde KW, Lohse GA (1966) *Die Käfer Mitteleuropas. Band 9. Cerambycidae, Chrysomelidae*. Goecke & Evers, Krefeld, 299 pp. (in german).

Freude H, Harde KW, Lohse GA (1981) *Die Käfer Mitteleuropas. Band 10. Bruchidae, Anthribidae, Scolytidae, Platypodidae, Curculionidae*. Goecke & Evers, Krefeld, 310 pp. (in german).

Fry B, Joern A, Parker PL (1978) Grasshopper food web analysis: use of carbon isotope ratios to examine feeding relationshipsamong terrestrial herbivores. *Ecology*, **59**, 498–506.

Futuyma DJ, Mitter C (1996) Insect-plant interactions: the evolution of component communities. *Philosophical Transaction Royal Society, Series B*, **351**, 1361–1366.

Futuyma DJ, Keese MC, Funk DJ (1995) Genetic constraints on macroevolution: the evolution of host affiliation in the leaf beetle genus Ophraella. *Evolution*, **49**, 797–809.

Garcia-Robledo C, Erickson DL, Staines CL, Erwin TL, Kress WJ (2013) Tropical plant-herbivore networks: reconstructing species interactions using DNA barcodes. *Public Library of Science ONE*, **8**, e52967.

Gillot C (2005) *Entomology*, 2nd edn. Springer, Dordrecht.

Grimaldi D, Engel MS (2005) *Evolution of the Insects*. Cambridge University Press, Cambridge, UK.

Gruev B, Tomov V (1984) *Coleoptera, Chrysomelidae. Part I. Fauna Bulgarica*, Aedibus Academiae Scientiarum Bulgaricae, Sofia, Hungary.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PHYML 3.0. *Systematic Biology*, **59**, 307.

Hajibabaei M, Shokralla S, Zhou X, Singer GAC, Baird DJ (2011) Environmental barcoding: a next-generation sequencing approach for biomonitoring applications using river Benthos. *Public Library of Science ONE*, **6**, e17497.

Hall TA (1999) BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95–98.

Harper GL, King RA, Dodd CS *et al.* (2005) Rapid screening of invertebrate predators for multiple prey DNA targets. *Molecular Ecology*, **14**, 819–827.

Hartley SE, Jones CG (1997) Plant chemistry and herbivory, or why the world is green. In: *Plant Ecology* (ed. Crawley MJ), pp. 284–324. Blackwell Science, Oxford, UK.

Hebert PDN, Ratnasingham S, deWaard JR (2003) Barcoding animal life: cytochrome coxidase subunit 1 divergences among closely related species. *Proceeding of the Royal Society B: Biological Science*, **270**, 96–99.

Heise W, Kubisz D, Babik W, Kajtoch Ł (2015) A three-marker DNA barcoding approach for ecological studies of xerothermic plants and herbivorous insects from central Europe. *Botanical Journal of Linnaean Society*, **177**, 576–592.

Hofreiter M, Poinar HN, Spaulding WG *et al.* (2000) A molecular analysis of ground sloth diet through the last glaciation. *Molecular Ecology*, **9**, 1975–1984.

Holechek JL, Vavra M, Pieper RD (1982) Botanical composition determination of range diets: a review. *Journal of Range Management*, **35**, 309–315.

Holecová M, Franc V (2001) Cervený (ekosozologický) zoznam chrobákov (Coleoptera) Slovenska. In: (eds Baláž D, Marhold K, Urban P) Cervený zoznam rastlín a živocíchov Slovenska. *Ochrana Prírody*, **20**(Suppl.), 111–128 (in czech).

Ibanez S, Manneville O, Miquel C *et al.* (2013) Plant functional traits reveal the relative contribution of habitat and food preferences to the diet of grasshoppers. *Oecologia*, **173**, 1459–1470.

Janišová M, Bartha S, Kiehl K, Dengler J (2011) Advances in the conservation of dry grasslands. Introduction to contributions from the 7th European Dry Grassland Meeting. *Plant Biosystematics*, **145**, 507–513.

Janz N, Nylin S, Wahlberg N (2006) Diversity begets diversity: host expansions and the diversification of plant-feeding insects. *BMC Evolutionary Biology*, **6**, 4.

Jermy T (1976) Insect-host-plant relationships – coevolution or sequential evolution? *Symposia Biologica Hungarica*, **16**, 109–113.

Jermy T (1984) Evolution of insect – host plant relationships. *American Naturalist*, **124**, 609–630.

Johnson SA, Nicolson SW (2001) Pollen digestion in flower-feeding Scarabaeidae: protea beetles (Cetoniini) and monkey beetles (Hopliini). *Journal of Insect Physiology*, **47**, 725–733.

Jolivet P (1998) *Interrelationship Between Insects and Plants*. CRC Press, Boca Raton, FL.

Jolivet P, Verma KK (2002) *Biology of Leaf Beetles*, Intercept, Andover, UK.

Jones RW (2001) Evolution of the host plant associations of the Anthonomus grandis species group (Coleoptera: Curculionidae): phylogenetic tests of various hypotheses. *Annals of the Entomological Society of America*, **94**, 51–58.

Jurado-Rivera JA, Vogler AP, Reid CAM, Petitpierre E, Gómez-Zurita J (2009) DNA barcoding insect–hostplant associations. *Proceeding of the Royal Society B: Biological Science*, **276**, 639–648.

Kajtoch Ł (2014) A DNA metabarcoding study of a polyphagous beetle dietary diversity: the utility of barcodes and sequencing techniques. *Folia Biologica (Krakow)*, **62**, 223–234.

Kajtoch Ł, Mazur MA (2015) The impact of environmental conditions on efficiency of host plant DNA barcoding for polyphagous beetles. *Environmental Entomology*. doi:10.1093/ee/nvv019.

Kajtoch Ł, Kubisz D, Lachowska-Cierlik D, Mazur MA (2013) Conservation genetics of endangered leaf-beetle *Cheilotoma musciformis* populations in Poland. *Journal of Insect Conservation*, **17**, 67–77.

Kajtoch Ł, Mazur M, Kubisz D, Mazur MA, Babik W (2014) Low effective population sizes and limited connectivity in xerothermic beetles: Implications for the conservation of an endangered habitat. *Animal Conservation*, **5**, 454–466.

Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, **30**, 772–780.

Kishimoto YK, Kamiya K, Meleng P *et al.* (2013) Wide host ranges of herbivorous beetles? Insights from DNA bar coding. *Public Library of Science ONE*, **8**, e74426.

Kitson JJN, Warren BH, VincentFlorens FB, Baider C, Strasberg D, Emerson BC (2013) Molecular characterization of trophic ecology within an island radiation of insect herbivores (Curculionidae: Entiminae: Cratopus). *Molecular Ecology*, **22**, 5441–5455.

Kubisz D, Kajtoch Ł, Mazur MA, Lis A, Holecová M (2012) Conservation genetics of highly isolated populations of xerothermic *Crioceris quatuordecimpunctata* (Coleoptera: Chrysomelidae). *Invertebrate Biology*, **131**, 333–344.

Kuntze R (1931) Vergleichende beobachtungen und betrachtungen über die xerotherme Fauna in Podolien, Brandenburg,

österreich und der schweiz. *Zeitschrift für Morphologie und Ökologie der Tiere, Berlin*, **21**, 629–690.

Lawrence JF, Ślipiński A, Seago AE, Thayer MK, Newton AF, Marvaldi AE (2011) Phylogeny of the Coleoptera based on morphological characters of adults and larvae. *Annales Zoologici*, **61**, 1–217.

Little DP (2014) A DNA mini-barcode for land plants. *Molecular Ecology Resources*, **14**, 437–446.

Matheson CD, Muller GC, Junnila A *et al.* (2008) A PCR method for detection of plant meals from the guts of insects. *Organisms Diversity & Evolution*, **7**, 294–303.

Matuszkiewicz W (2005) *Przewodnik do Oznaczania Zbiorowisk Roślinnych Polski [Manual to Identification of Plant Communities in Poland]*. Scientific Publisher, Warsaw, Poland (in polish).

Mazur M (2001) *Ryjkowce Kserotermiczne Polski (Curculionoidea: Nemonychidae, Attelabidae, Apionidae, Curculionidae). Studium Zoogeograficzne [Xerothermic Weevils of Poland (Curculionoidea: Nemonychidae, Attelabidae, Apionidae, Curculionidae). Zoogeographic Studies]*. Faunistic Monographs, Institute of Systematics and Evolution of Animals Polish Academy of Sciences, Krakow, Poland (in polish).

Mazur M (2002) The distribution and ecology of weevils (Coleoptera: Nemonychidae, Attelabidae, Apionidae, Curculionidae) in western Ukraine. *Acta Zoologica Cracoviensia*, **45**, 213–244.

Mazur MA (2006) Weevils (Coleoptera: curculionoidea: anthribidae, Apionidae, Curculionidae, Rhynchitidae) of selected excavations of Opole Silesia. In: *Biodiversity of Quarries and Pits* (eds Nowak A, Hebda G), pp. 145–163. Opole Scientific Society, Opole, Poland.

Mazur M, Kubisz D (2013) *Rozmieszczenie i Migracje Kserotermicznych Chrzaszczy (Coleoptera) w Dolinie Wisly [Distribution and Migration of the Xerothermic Beetles (Coleoptera) in the Vistula River Valley]*. Faunistic Monographs, Institute of Systematics and Evolution of Animals Polish Academy of Sciences, Krakow, Poland (in polish).

Mazur MA, Kubisz D, Kajtoch Ł (2014) Restricted geographic distribution and low genetic distinctiveness of steppic *Crioceris quinquepunctata* (Coleoptera: Chrysomelidae) populations in central-east Europe. *Entomologica Fennica*, **25**, 103–111.

Metcalf RL (1986) Coevolutionary adaptations of rootworm beetles (Coleoptera: Chrysomelidae) to cucurbitacins. *Journal of Chemical Ecology*, **12**, 1109–1124.

Miller JS, Wenzel JW (1995) Ecological characters and phylogeny. *Annual Review of Entomology*, **40**, 389–415.

Mitter C, Brooks DR (1983) Phylogenetic aspects of coevolution. In: *Coevolution* (eds Futuyma DJ, Slatkin S), pp. 65–98. Sinaner Associates, Sunderland, UK.

Mitter C, Farrell B (1991) Macroevolutionary aspects of insect-plant relationships. In: *Insect-Plant Interactions, v. 3*. (ed. Bemays E), pp. 35–78. CRC Press, Boca Raton, Florida.

Mitter C, Farrell B, Futuyma DJ (1991) Phylogenetic studies of insect-plant interactions: insights into the genesis of diversity. *Trends in Ecology & Evolution*, **6**, 290–293.

Moritz C, Cicero C (2004) DNA barcoding: promise and pitfalls. *Public Library of Science Biology*, **2**, e354.

Nazarenko V (2009) Weevils of the branch of the Ukrainian Steppe Natural Reserve "Mykhajlivska tsilyna" and adjacent territories. *Vestnik Zoologii*, **22**(Suppl.), 36–50 (in ukrainian).

Novotny V, Basset Y, Miller SE *et al.* (2002) Low host specificity of herbivorous insects in a tropical forest. *Nature*, **416**, 841–844.

Novotny V, Drozd P, Miller SE *et al.* (2006) Why are there so many species of herbivorous insects in tropical rainforests? *Science*, **313**, 1115–1118.

Novotny V, Miller SE, Basset Y *et al.* (2007) Low beta diversity of herbivorous insects in tropical forests. *Nature*, **448**, 692–696.

Nylander JAA (2004) *MRMODELTEST v2. Program Distributed by the Author*. Evolutionary Biology Centre, Uppsala University, Sweden.

Oberprieler RG, Marvaldi AE, Anderson RS (2007) Weevils, weevils, weevils everywhere. *Zootaxa*, **1668**, 491–520.

Otte D, Joern A (1976) On feeding patterns in desert grasshoppersand the evolution of specialized diets. *Proceedings of the Academy of Natural Sciences of Philadelphia*, **128**, 89–126.

Pawłowski J, Kubisz D, Mazur M (2002) Coleoptera. In: *Red List of Threatened Animals in Poland* (ed. Glowacinski Z), pp. 88–110. Polish Academy of Sciences, Institute of Nature Conservation, Kraków, Poland.

Petitpierre E, Segarra C (1985) Chromosomal variability and evolution in Chrysomelidae (Coleoptera), particularly that of Chrysomelinae and Palearctic Alticinae. *Entomography*, **3**, 403–426.

Pinzón-Navarro SP, Jurado-Rivera JA, Gomez-Zurita J, Lyal CHC, Vogler AP (2010) DNA profiling of host herbivore interactions in tropical forests. *Ecological Entomology*, **35**, 18–32.

Pons J, Barraclough TG, Gomez-Zurita J *et al.* (2006) Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology*, **55**, 595–609.

Preuss H (1912) Die pontischen Pflanzenbestande im Weichselgebiet. *Beitrage zu Naturdenkmalpflege*, **2**, 350–517.

Rambaut A (2009) *FigTree v1.3.1: Tree Figure Drawing Tool*. Available from http://treebioedacuk/software/figtree/. Accessed 2012 April 20.

Sandholm HA, Price RD (1962) Field observations on the nectar feeding habits of some Minnesota mosquitoes. *Mosquito News*, **22**, 846–849.

Schmitt M, Rönn T (2011) Types of geographical distribution of leaf beetles (Chrysomelidae) in Central Europe. *ZooKeys*, **157**, 131–158.

Schultz JC (1988) Many factors influence the evolution of herbivore diets, but plant chemistry is central. *Ecology*, **69**, 896–897.

Sheppard SK, Harwood JD (2005) Advances in molecular ecology: tracking trophic links through predator-prey food-webs. *Functional Ecology*, **19**, 751–762.

Simon C, Frati F, Bechenbach A, Crespi B, Liu H, Flock P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequence and compilation of conserved polymerase chain reaction primers. *Annals Entomological Society America*, **87**, 651–701.

Soininen EM, Valentini A, Coissac E *et al.* (2009) Analysing diet of small herbivores: the efficiency of DNA barcoding coupled with high-throughput pyrosequencing for deciphering the composition of complex plant mixtures. *Frontiers in Zoology*, **6**, 16.

Swofford DL (2002) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Sinauer Associates, Sunderland, Massachusetts.

Symondson WOC (2002) Molecular identification of prey in predatordiets. *Molecular Ecology*, **11**, 627–641.
Szymczakowski W (1960) Materiały do poznania kserotermofilnej fauny chrząszczy Wyżyny Małopolskiej [Materiale to knowledge of xerothermophilous fauna of beatles on Małopolska Upland]. *Polish Journal of Entomology*, **30**, 173–242. (in polish).
Szymczakowski W (1965) Materiały do poznania chrząszczy (Coleoptera) siedlisk kserotermicznych Polski [Materiale to knowledge of beatles (Coleoptera) xerothermic plant communities in Poland]. *Polish Journal of Entomology*, **35**, 225–257. (in polish).
Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three noncoding regions of chloroplast DNA. *Plant Molecular Biology*, **17**, 1105–1109.
Taberlet P, Coissac E, Pompanon F *et al.* (2007) Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. *Nucleic Acids Research*, **35**, e14.
Taberlet P, Coissac E, Pompanon F, Brochmann C, Willerslev E (2012) Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*, **21**, 2045–2050.
Thorsteinson AJ (1960) Host selection in phytophagous insects. *Annual Review Entomology*, **5**, 193–218.
Valentini A, Miquel C, Nawaz MA *et al.* (2009a) New perspectives in diet analysis based on DNA barcoding and parallel pyrosequencing: the trnL approach. *Molecular Ecology Resources*, **9**, 51–60.
Valentini A, Pompanon F, Taberlet P (2009b) DNA barcoding for ecologists. *Trends in Ecology & Evolution*, **24**, 110–117.
Wanat M, Mokrzycki T (2005) A new checklist of the weevils of Poland (Coleoptera: Curculionoidea). *Genus*, **16**, 69–117.
Warchałowski A (1991) *Chrysomelidae. Leaf Beetles (Insecta: Coleoptera). Part II (Subfamilies: Clythrinae and Cryptocephalinae).* Fauna of Poland, Warszawa, Poland (in polish).
Warchałowski A (1976) Biogeographische Studien über die Blattkäfer der Pontischen Provinz (Coleoptera, Chrysomelidae). *Polish Journal of Entomology*, **46**, 29–94.
Ward LK, Hackshaw A, Clarke RT (2003) Do food-plant preferences of modern families of phytophagous insects and mites reflect past evolution with plants. *Biological Journal of the Linnean Society*, **78**, 51–83.
Wąsowska M (2006) Chrysomelid communities (Chrysomelidae, Coleoptera) of xerothermic grasslands (*Inuletum ensifoliae*) in the Wyzyna Miechowska Uplands (Central Poland). *Biologia, Bratislava*, **61**, 565–572.
Wesche K, Krause B, Culmsee H, Leuschner C (2012) Fifty years of change in Central European grassland vegetation: large losses in species richness and animal-pollinated plants. *Biological Conservation*, **150**, 76–85.
Zając A, Zając M (eds) (2001) *Atlas Rozmieszczenia Roslin Naczyniowych w Polsce. [Distribution Atlas of Vascular Plants in Poland].* Laboratory of Computer Chorology, Institute of Botany, Jagiellonian University, Krakow, Poland (in polish).
Zhi-Quiang Z (2013) Phylum Athropoda. [In:] Zhi-Quiang Z (Ed.) Animal biodiversity: an outline of higher-level classification and survey of taxonomic richness (Addenda 2013). *Zootaxa*, **3703**, 17–26.

All authors designed the research. W.H. collected plant samples. D.K., M.A.M. and Ł.K. collected beetle samples. Ł.K. and W.B. performed molecular analysis. Ł.K. and W.B. wrote the manuscript. All authors contributed to the final version of the manuscript.

Data accessibility

Raw DNA sequences: GenBank accessions: rbcL: KJ746116–KJ746208; matK: KJ746209–KJ746322; trnL: KJ746323–KJ746436; COI: KP306793-KP306891.

Final plant barcode assemblies available as supporting information in separate article (Heise *et al.* 2015, Botanical Journal of Linnaean Society; http://onlinelibrary.wiley.com/doi/10.1111/boj.12261/suppinfo).

Alignments of COI sequences for phylogenetics and maximum-likelihood COI trees files; alignments of rbcL sequences for phylogenetics and maximum-likelihood rbcL trees file; and Illumina trnL and rbcL sequences files and sampling locations (table and Google maps file): Dryad: doi:10.5061/dryad.26h4v.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Maximum Likelihood phylogenetic tree reconstructed for rbcL sequences of steppic plants (green) and sequences obtained from beetles: weevils (blue) and leaf beetles (red).

Fig. S2 Dendrogram of similarities between four selected groups of steppic beetles (leaf beetles and weevils, both polyphagous and not-polyphagous) in respect to their feeding on particular plant species, constructed using Cluster Analysis.

Table S1 Effects of host plant identification for steppic beetles executed with the use of Sanger sequencing of two barcodes (rbcL gene and trnL intron) and MEGABLAST search against barcode database of xerothermic plants from Poland.

Table S2 Effects of host plant identification for 15 selected species of steppic beetles and a sample of 8 plant species (*Eryngium planum, Inula ensifolia, Onobrychis viciifolia, Adonis vernalis, Salvia pratensis, Rosa canina, Arenaria serpyllifolia, Elymus repens*; validation) executed using Illumina sequencing of two barcodes (rbcL gene and trnL intron) and a MEGABLAST search against the barcode database of xerothermic plants from Poland.

Appendix S1 Sanger sequencing and host plant identification.

Appendix S2 Validation of Illumina approach.

Appendix S3 Illumina sequencing and host plant identification.

Appendix S4 Limitations of host plant barcoding.