ARTHROPOD SYSTEMATICS
& PHYLOGENY

76(1): 45–58
14.5.2018

SENCKENBERG

© Senckenberg Gesellschaft für Naturforschung, 2018.

New molecular markers resolve the phylogenetic position of the enigmatic wood-boring weevils Platypodinae (Coleoptera: Curculionidae)

Sigrid Mugu, Dario Pistone & Bjarte H. Jordal *

Department of Natural History, The University Museum, University of Bergen, PO Box 7800, NO-5020 Bergen, Norway; Sigrid Mugu [syky33@hotmail.com]; Dario Pistone [Dario.Pistone@uib.no]; Bjarte Jordal * [bjarte.jordal@uib.no] — * Corresponding author

Accepted 06.xii.2017.
Published online at www.senckenberg.de/arthropod-systematics on 30.iv.2018.
Editors in charge: Rudolf Meier & Klaus-Dieter Klass

Abstract. The precise phylogenetic position of the weevil subfamily Platypodinae continues to be one of the more contentious issues in weevil systematics. Morphological features of adult beetles and similar ecological adaptations point towards a close relationship with the wood boring Scolytinae, while some recent molecular studies and larval morphology have indicated a closer relationship to Dryophthorinae. To test these opposing hypotheses, a molecular phylogeny was reconstructed using 5,966 nucleotides from ten gene fragments. Five of these genes are used for the first time to explore beetle phylogeny, i.e. the nuclear protein coding genes PABP1, UBA5, Arr2, TPI, and Iap2, while five markers have been used in earlier studies (28S, COI, CAD, ArgK, and EF-1α). Bayesian, maximum likelihood and parsimony analyses of the combined data strongly support a monophyletic Curculionidae (the advanced weevils with geniculate antennae), where Brachycerinae, Platypodinae, and Dryophthorinae formed the earliest diverging groups. Dryophthorinae and core Platypodinae were sister groups with high support, with the contentious genera *Mecopelmus* Blackman, 1944 and *Coptonotus* Chapuis, 1873 placed elsewhere. Other lineages of wood boring weevils such as Scolytinae, Cossoninae, and Conoderinae were part of a derived, but less resolved, clade forming the sister group to Entiminae. Resolution among major curculionid subfamilies was ambiguous, emphasizing the need for large volumes of data to further improve resolution in this most diverse section of the weevil tree.

Key words. Weevils, molecular phylogeny, Platypodinae, Scolytinae, Dryophthorinae, ambrosia beetles, TPI, UBA5, PABP1, Arrestin2, Iap2.

1. Introduction

The weevil superfamily Curculionoidea represents one of the most diverse groups of insects, with more than 60,000 described species (Oberprieler et al. 2007). Classification of the group has changed considerably over the past centuries, as can be expected for such a tremendously diversified group. Recent revisions of higher taxa (Alonso-Zarazaga & Lyal 1999; Oberprieler et al. 2007) have highlighted considerable uncertainty tied to the placement and rank of certain taxa, but have also pointed towards a gradually unified classification, largely founded on, and confirmed by, recent phylogenetic analyses (Kuschel 1995; Marvaldi et al. 2002; McKenna et al. 2009; Jordal et al. 2011; Haran et al. 2013; Gillett et al. 2014; Gunter et al. 2015).

There is now a certain consensus that orthocerous weevil families (weevils with straight antennae) form a variety of older diverging lineages, including Nemonychidae, Anthribidae, Attelabidae, Belidae, Caridae, and Brentidae. Most of the controversy is therefore associated with the placement and rank of the advanced weevils which are characterized by geniculate antennae – the megadiverse family Curculionidae sensu Oberprieler et al. (2007) (Fig. 1). The generally low phylogenetic resolution obtained so far may be a consequence of limited molecular data per taxon unit, as well as the high number of species, with species-rich clades requiring larger data volumes to obtain resolution. The type of data used in previous analyses has mainly been of ribosomal or mitochondrial origin, with no more than five nuclear protein coding genes applied to date (Farrell et al. 2001; McKenna et al. 2009; Jordal et al. 2011; Riedel et al. 2016). A commonly used ribosomal marker, the 18S gene, has

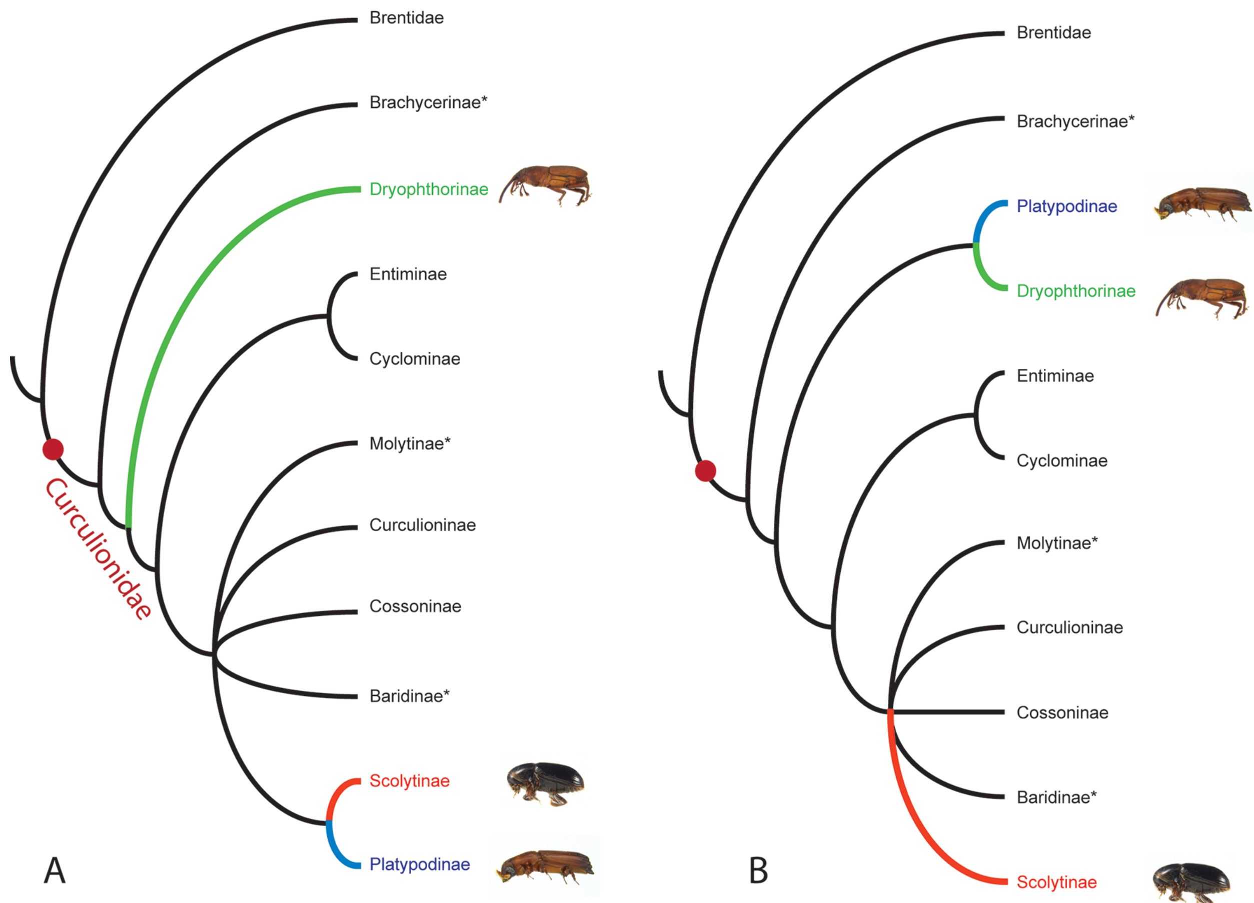

Fig. 1. Two main hypotheses on relationships in the advanced weevils, using Brentidae as outgroup: **A**: Proposed by Kuschel (1995) and partially supported by mixed morphological and molecular data in Farrell (1998), Marvaldi et al. (2002), and Jordal et al. (2011). **B**: Proposed by Marvaldi (1997) and supported by molecular data in McKenna et al. (2009), Haran et al. (2013), and Gillett et al. (2014). Subfamilies marked by * as broadly defined by Oberprieler et al. (2007).

a low substitution rate and, hence, contains very limited information for weevil phylogenetics (Farrell 1998). Additional markers are therefore much needed to enable further resolution of the weevil tree.

Perhaps the most contentious issue in weevil phylogenetics is the placement of the wood boring and fungus-farming subfamily Platypodinae (Jordal et al. 2014; Jordal 2015). These beetles share a functional niche with 11 fungus-farming lineages in another weevil subfamily, Scolytinae (Hulcr et al. 2015). These all live in nutritional symbiosis with Microascales and Ophiostomatales ambrosia fungi and are therefore generally referred to as ‘ambrosia beetles’ (Beaver 1989). Platypodine and scolytine ambrosia beetles excavate tunnel systems in dead trees into which they inoculate fungal spores and cultivate small fungal gardens in the wood; this serves as the only food source for their larvae. Fungus farming is a truly unique evolutionary innovation seen elsewhere only in one clade of ants and one clade of termites (Mueller & Gerardo 2002).

The wood boring behaviour that characterizes bark and ambrosia beetles is generally associated with a substantial reduction in rostrum length and strengthened tibial spines, a feature also seen in some other wood boring weevils such as many Cossoninae and the conoderine tribe Campyloscelini (Jordal et al. 2011; Kirkendall et al. 2014). Wood boring taxa have often been placed close to each other in classifications due to morphological similarities (Blandford 1897; Kuschel 1995; Kuschel et al. 2000; Oberprieler et al. 2014; see Fig. 1A), in particular, Platypodinae and Scolytinae (Wood 1986; Morimoto & Kojima 2003). However, morphological data (Lyal 1995; Marvaldi 1997) and recent molecular phylogenetic studies (McKenna et al. 2009; Gillett et al. 2014; Gunter et al. 2015) have indicated that this may not reflect evolution, but adaptation to similar life styles. Some large-scale molecular studies have rather suggested a close, but weakly supported, relationship between Platypodinae and Dryophthorinae (McKenna et al. 2009; Haran et al. 2013; Gillett et al. 2014), in particular agreement with larval and pupal morphology (Marvaldi 1997). Both types of data also suggest that platypodines and dryophthorines are advanced weevils, forming the first diverging clade after the origin of Brachycerinae (Fig. 1B). All three subfamilies (*sensu* Oberprieler et al. 2007) have therefore been ranked as families by some authors, as opposed to subfamilies, and placed outside a more narrowly defined Curculionidae (*sensu* Thompson 1992; Zimmerman 1993, 1994; Alonso-Zarazaga & Lyal 1999).

Table 1. Primers used in PCR and sequencing, and the optimal annealing temperature.

Gene	Primer sequence		Annealing temperature
Large ribosomal subunit (28S)	(S3690) (A4285) (A4394)	GAGAGTTMAASAGTACGTGAAAC CTGACTTCGTCCTGACCAGGC TCGGAAGGAACCAGCTACTA	55°C (45s)
Arginine kinase (ArgK)	(forB2) (revB1) (LTrev2)	GAYTCCGGWATYGGWATCTAYGCTCC TCNGTRAGRCCCATWCGTCTC GATKCCATCRTDCATYTCCTTSACRGC	50°C (45s)
Arrestin2 (Arr2)	(F) (R)	CGYGARGAGGAYGARGTYATGGG ACCATSGTRACYTCGCAATGYTGCAC	52°C (30s)
Carbamoyl-phosphate synthetase 2 (CAD)	(forB2) (for4) (rev1mod)	GARAARGTNGCNCCNAGTATGGC TGGAARGARGTBGARTACGARGTGGTYCG GCCATYRCYTCBCCYACRCTYTTCAT	50°C (45s)
Cytochrome oxidase I (COI)	(S1718) (A2237) (A2411)	GGAGGATTTGGAAATTGATTAGTTCC CCGAATGCTTCTTTTTTACCTCTTTCTTG GCTAATCATCTAAAAACTTTAATTCCWGTWG	46°C (45s)
Elongation factor 1 alpha (EF-1α)	(S149) (A1043) (A754)	ATCGAGAAGTTCGAGAAGGAGGCYCARGAAATGGG GTATATCCATTGGAAATTTGACCNGGRTGRTT CCACCAATTTTGTAGACATC	58–44°C*
Inhibitor of apoptosis 2 (Iap2)	(F2) (R)	CCATCKGGCRTGYTCYGTCCAWGGATC TGGAAYTAYGGRGACCAAGTRATGGC	52°C (30s)
Polyadenylate binding protein (PABP1)	(F) (R)	CCRATTCGYATYATGTGGTC GAARGCRACAAAWCCRAAWCC	50°C (30s)
Triosephosphate isomerase (TPI)	(46F) (615R)	GGTGGHAACTGGAARATGAACGG CKGARCCYCCRTATTGRATTC	50°C (45s)
Ubiquitin-like modifier activating enzyme 5 (UBA5)	(F) (R)	TTGGKAGYGTAACWGCRGAAATG ATATGGCCWGARACSGCRTTTTC	48°C (30s)

In order to establish a more robust resolution in the weevil phylogeny, we added five new nuclear protein coding genes to the phylogenetic analysis. The new markers were originally screened and optimized for bark beetle phylogenetics (Pistone et al. 2016), and we have tested their usefulness for a broader range of weevil taxa. Based on new sequence data, we tested the hypothesis that Platypodinae is the sister group to Dryophthorinae, using a largely unbiased taxon sampling that represents most major groups of advanced weevils.

2. Materials and methods

Samples included 72 species of 15 different subfamilies in the family Curculionidae, *sensu* Alonso-Zarazaga & Lyal (1999), or 9 subfamilies *sensu* Oberprieler et al. (2007). Ten species of Anthribidae, Attelabidae, Apionidae and Brentidae were included as outgroup taxa (Table 2). DNA was extracted from a leg for each of the larger species, or head and pronotum for smaller species, using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

PCR (Polymerase Chain Reaction) was used to amplify gene fragments prior to Sanger sequencing. DNA sequences were obtained from ten genes, five of these have not previously, or only rarely, been used in beetle phylogenetics: Triose phosphate isomerase (TPI), Arrestin 2 (Arr2), Inhibitor of apoptosis 2 (Iap2), Ubiquitin-like modifier-activating enzyme 5 (UBA5), and Polyadenylate-binding protein 1 (PABP1).

TPI is a key enzyme of the glycolysis pathway (Wierenga et al. 2010) and has occasionally been used in phylogenetic analyses of insects (Hardy 2007; Wiegmann et al. 2009; McKenna & Farrell 2010).

Arr2 is a mediator protein involved in the sensitization of G-protein-coupled receptors and in other signalling pathways (Gurevich & Gurevich 2006). Molecular characterization of this gene in *Maruca vitrata* (Lepidoptera: Crambidae) has demonstrated congruence with basal holometabolan relationships and could potentially be valuable as a phylogenetic marker (Chang & Ramasamy 2013).

Iap2 is a member of the inhibitor of apoptosis protein family, mainly involved in regulation of caspase activity ensuring cell survival (Leulier et al. 2006; Huh et al. 2007). Iap2 in particular is required for the innate immune response to Gram-negative bacterial infections (Rajalingam et al. 2006).

UBA5 is an E1 enzyme responsible for the activation of ubiquitin-fold modifier 1 protein (Umf1) by forming a high-energy thioester bond (Komatsu et al. 2004; Dou et al. 2005; Bacik et al. 2010; Gavin et al. 2014). Ubiquitination, including the process of post-translational modification or addition of ubiquitin to a protein, is carried out by activation, conjugation and ligation performed by three ubiquitin-modifier classes of enzymes (E1, E2 and E3 respectively). Information regarding UBA5 in insects is very limited.

PABP1 is known as the Poly (A) binding protein 1, which plays a crucial role for the messenger RNA transportation from the nucleus (Apponi et al. 2010). This protein has a conserved structure in the Metazoa (Smith et al. 2014). PABP1 has not been used in phylogenetic studies

Table 2. Species included in this study and their GenBank accession numbers. The code represents the DNA specimen voucher at the University Museum of Bergen. The classification scheme follows Alonso-Zarazaga et al. (1999).

FAMILY	TRIBE	SPECIES	CODE	COUNTRY	CO1	EF-1α	28S	CAD	ArgK	PABP1	UBA5	Iap2	Arrestin2	TPI
Anthribidae	indet.	Anthribidae indet. sp2	Antrib02	Cameroon	HQ883608	HQ883696	–	HQ883765	HQ883841	KU041907	–	KU042009	KU163337	KU041951
Anthribidae	indet.	Anthribidae indet. sp3	Antrib03	Tanzania	KU041889	KU041896	–	KU041884	–	–	–	KU042010	KU163338	–
Anthribidae	indet.	Anthribidae indet. sp3	Antrib04	Madagascar	–	KU041897	–	KU041885	–	KU041908	KU041975	KU042011	–	–
Attelabidae	Apoderini	*Apoderus coryli*	AtApo01	Russia	HQ883609	–	HQ883528	–	HQ883842	KU041909	–	–	–	–
Attelabidae	Apoderini	*Apoderus jekeli*	AtApo02	Russia	HQ883610	HQ883697	HQ883529	–	HQ883843	–	–	–	KU163339	–
Apionidae	Apionini	*Apion (Apion) cruentatum*	BrApi03	Norway	KU041890	KU041898	KU041867	–	–	KU041911	–	–	–	–
Apionidae	Apionini	*Apion (Perapion) curtirostre*	BrApi01	Norway	HQ883612	HQ883698	HQ883531	HQ883767	–	KU041910	–	–	–	KU041952
Brentidae	(Brentinae)	Brentinae indet. sp1	BrBre01	Sarawak	HQ883613	HQ883699	HQ883532	–	HQ883845	KU041912	KU041976	–	–	–
Brentidae	(Brentinae)	Brentinae indet. sp2	BrBre02	Cameroon	HQ883614	HQ883700	HQ883533	HQ883768	HQ883846	–	KU041977	–	KU163340	KU041953
Brentidae	(Brentinae)	Brentinae indet. sp3	BrBre03	Cameroon	KU041891	–	KU041868	–	KU041878	KX160751	KX160704	–	KX160641	–
Curculionidae														
SUBFAMILY														
Ceutorhynchinae	Ceutorhynchini	*Zacladus affinis*	CeZac01	Norway	HQ883621	HQ883706	HQ883540	HQ883772	HQ883853	KU041917	KU041980	KU042016	–	KU041959
Ceutorhynchinae	Phytobiini	*Rhinoncus pericarpius*	CeRhi01	Norway	HQ883620	–	HQ883539	HQ883771	HQ883852	KU041916	–	KU042015	–	KU041958
Conoderinae	Campyloscelini	Campyloscelini indet.	CdCod02	Cameroon	HQ883616	HQ883702	HQ883535	HQ883769	HQ883848	–	KU041978	KU042012	–	KU041954
Conoderinae	Campyloscelini	*Homoeometamelus* sp1	CsXxA01	Uganda	HQ883643	HQ883723	HQ883558	HQ883785	HQ883872	KU041926	–	KU042023	–	–
Conoderinae	Campyloscelini	*Homoeometamelus* sp2	CsXxA02	Cameroon	–	–	–	–	–	KU041927	KU041990	KU042024	KU163349	–
Conoderinae	Campyloscelini	*Homoeometamelus* sp3	CsXxA03	Cameroon	–	–	–	–	–	KU041928	KU041991	KU042025	KU163350	–
Conoderinae	Campyloscelini	*Scolytoproctus* sp.	CdSpr01	South Africa	HQ883618	HQ883704	HQ883537	HQ883770	HQ883850	KU041914	–	KU042013	–	KU041956
Conoderinae	Conoderini	Conoderini sp.	CdZyg01	Russia	HQ883619	HQ883705	HQ883538	–	HQ883851	KU041915	KU041979	KU042014	KU163342	KU041957
Conoderinae	Conoderini	*Metialma* sp.	CdMet	South Africa	HQ883617	HQ883703	HQ883536	–	HQ883849	KU041913	–	–	KU163341	KU041955
Coptonotinae	Coptonotini	*Coptonotus cyclopus*	CpCop01	CR	HQ883624	–	–	HQ883774	HQ883856	KU041918	–	–	KU163344	KU041960
Coptonotinae	Mecopelmini	*Mecopelmus zeteki*	MeMec01	Panama	HQ883663	HQ883735	HQ883574	HQ883802	HQ883892	KU041939	–	–	KU163353	–
Cossoninae	Araucariini	*Araucarius major*	CsAru02	Argentina	AY040285	HQ883711	AF308350	–	HQ883860	KU041922	KU041985	KU042020	KU163347	–
Cossoninae	Araucariini	*Araucarius minor*	CsAru01	Argentina	AF375307	AF308346	AF308351	–	HQ883859	–	KU041984	KU042019	KU163346	–
Cossoninae	Araucariini	*Coptocorynus* sp.	CsCpt02	PNG	HQ883631	HQ883714	HQ883546	HQ883776	HQ883862	KU041923	KU041986	–	–	KU041963
Cossoninae	Araucariini	*Xenocnema* sp.	CsXen01	Australia	HQ883642	HQ883722	HQ883557	–	–	KU041925	KU041989	–	–	–
Cossoninae	Cossonini	*Mesites fusiformis*	CsMes01	Spain	EU191838	EU191870	HQ883549	HQ883778	HQ883865	KX160754	–	–	–	KX160555
Cossoninae	Onycholipini	*Pselactus* sp.	CsPse01	Madeira	EU191839	EU191871	HQ883552	HQ883780	HQ883868	KX160755	KX160706	KX160628	KX160643	KX160556
Cossoninae	Onycholipini	*Pseudostenocelis* sp.	CsPsc01	PNG	HQ883636	HQ883717	HQ883551	HQ883779	HQ883867	KU041924	KU041987	KU042021	KU163348	–
Cossoninae	Rhyncolini	*Rhyncolus elongatus*	CsRhy02	Norway	–	KU041899	KU041869	–	–	–	KU041988	KU042022	–	–
Cryptorhynchinae	Cryptorhynchinae	Cryptorhynchinae indet sp1	Crh_sp1	South Africa	HQ883627	–	–	–	–	KU041920	KU041982	–	–	KU041961
Cryptorhynchinae	Cryptorhynchini	Cryptorhynchini indet. sp2	Crh_sp2	Cameroon	HQ883628	HQ883710	HQ883543	–	HQ883858	KU041921	KU041983	KU042018	–	KU041962
Curculioninae	Tychiini	*Sibinia* sp.	CuSib01	South Africa	HQ883649	HQ883725	HQ883563	–	HQ883878	KU041930	–	–	–	–
Dryophthorinae	indet.	Dryophthorinae indet. sp1	Dryoph01	Madagascar	KU041892	KU041900	KU041870	–	KU041879	KU041931	KU041994	–	–	KU041966
Dryophthorinae	indet.	Dryophthorinae indet. sp2	Dryoph02	Madagascar	KU041893	KU041901	KU041871	–	KU041880	KU041932	KU041995	–	–	–
Dryophthorinae	Rhynchophorini	*Rhynchophorus cruentatus*	Dryoph05	Spain	–	KU041903	KU041873	KU041886	KU041882	KU041934	–	KU042029	–	KU041967

Table 2 continued.

SUBFAMILY	TRIBE	SPECIES	CODE	COUNTRY	CO1	EF-1α	28S	CAD	ArgK	PABP1	UBA5	Iap2	Arrestin2	TPI
Dryophthorinae	Sitophilini	*Sitophilus oryzae*	Dryoph06	Norway	KU041894	–	KU041874	–	–	–	KU041997	–	–	–
Dryophthorinae	Sitophilini	*Sitophilus* sp.	Dryoph04	Madagascar	–	KU041902	KU041872	–	KU041881	KU041933	KU041996	KU042028	–	–
Entiminae	Otiorhynchini	*Otiorhynchus auropunctatus*	EnOti01	Norway	HQ883652	HQ883728	HQ883567	–	HQ883883	KU041936	–	–	–	–
Entiminae	Otiorhynchini	*Otiorhynchus sulcatus*	EnOti02	Norway	–	KU041904	KU041875	KU041887	–	KU041937	–	KU042031	–	–
Entiminae	Polydrusini	*Polydrusus cervinus*	EnPol01	Norway	HQ883653	HQ883729	HQ883568	HQ883793	HQ883884	–	–	–	–	–
Entiminae	Sitonini	*Chlorophanus sibiricus*	EnChl01	Russia	HQ883651	HQ883727	HQ883566	–	HQ883882	KU041935	KU041998	KU042030	–	–
Entiminae	Sitonini	*Sitona lineatus*	EnSit01	Norway	–	KU041905	KU041876	KU041888	KU041883	–	–	–	–	–
Erirhininae	Erirhinini	*Himasthlophallus flagellifer*	ErHim01	Russia	HQ883654	HQ883730	HQ883569	–	–	KU041938	–	–	–	–
Lixinae	Lixini	*Larinus* sp.	ClLar01	Russia	HQ883622	HQ883707	HQ883541	HQ883773	HQ883854	KX160752	KX160705	KX160625	–	–
Lixinae	Lixini	*Lixus* sp.	ClLix01	Russia	HQ883623	HQ883708	HQ883562	–	HQ883855	–	KU041981	KU042017	KU163343	–
Molytinae	Amorphocerini	*Amorphocerus rufipes*	MoAmo01	South Africa	HQ883664	HQ883736	HQ883575	HQ883803	HQ883893	KU041940	KU041999	KU042033	–	KU041969
Molytinae	Amorphocerini	*Porthetes hispidus*	MoPor01	South Africa	HQ883666	HQ883737	HQ883577	HQ883805	HQ883895	KX160765	–	KX160634	KX160650	–
Molytinae	Hylobiini	*Hylobius piceus*	MoHyl01	Norway	HQ883665	–	HQ883576	HQ883804	HQ883894	KU041941	KU042000	KU042034	–	–
Molytinae	indet.	Molytinae indet sp.	MoXxx01	Cameroon	HQ883667	HQ883738	HQ883578	HQ883806	HQ883896	KU041942	KU042001	KU042035	–	KU041970
Molytinae	Psepholacini	*Psepholax* sp.	Crh_Psx01	PNG	HQ883626	HQ883709	HQ883542	HQ883775	HQ883857	KU041919	–	–	KU163345	–
Platypodinae	Platypodini	*Platypus impressus*	PlPla07	Tanzania	–	–	–	–	–	KX160766	KX160714	KX160635	–	KX160564
Platypodinae	Platypodini	*Teloplatypus* sp1	PlTel01	CR	–	–	–	–	–	KU041943	KU042002	–	KU163354	–
Platypodinae	Platypodini	*Teloplatypus* sp2	PlTel02	CR	HQ883674	HQ883743	HQ883583	HQ883814	HQ883904	KU041944	KU042003	–	–	–
Platypodinae	Platypodini	*Triozastus marshalli*	PlTri02	Cameroon	KU041895	–	–	–	–	KX160767	KX160715	–	KX160651	KX160565
Platypodinae	Tesserocerini	*Cenocephalus* sp.	TsCen01	CR	HQ883682	HQ883751	HQ883593	HQ883826	HQ883912	KU041945	KU042004	–	KU163355	–
Platypodinae	Tesserocerini	*Chaetastus tuberculatus*	TsCha02	Cameroon	HQ883684	HQ883753	HQ883595	HQ883828	HQ883914	KX160775	–	–	KX160654	–
Platypodinae	Tesserocerini	*Diapus pusillimus*	TsDia01	PNG	–	–	–	–	–	KU041946	–	KU042036	–	KU041971
Platypodinae	Tesserocerini	*Diapus unispineus*	TsDia02	PNG	HQ883685	HQ883754	HQ883596	HQ883829	HQ883915	KU041947	KU042005	KU042037	–	KU041972
Platypodinae	Tesserocerini	*Genyocerus exilis*	TsGen02	Sarawak	HQ883686	HQ883755	HQ883597	HQ883830	HQ883916	KU041948	–	KU042038	KU163356	–
Platypodinae	Tesserocerini	*Notoplatypus elongatus*	TsNot01	Australia NSW	HQ883688	HQ883757	HQ883599	HQ883832	HQ883918	–	KU042006	–	–	–
Platypodinae	Tesserocerini	*Spathidicerus nobilis*	TsSpa01	PNG	–	KU041906	KU041877	–	–	KU041949	KU042007	KU042039	KU163357	KU041973
Platypodinae	Tesserocerini	*Tesserocerus ericerus*	TsTes01	CR	HQ883691	HQ883760	HQ883602	HQ883834	HQ883920	KU041950	KU042008	KU042040	KU163358	KU041974
Scolytinae	Dryocoetini	*Dryocoetes alni*	DrDry02	Norway	AF438508	AF439742	–	–	JX263918	MF771641	KU041993	KU042027	KU163352	KU041965
Scolytinae	Hexacolini	*Micoborus angustus*	CtMic03	Cameroon	HQ883645	–	HQ883560	HQ883788	HQ883874	KU041929	KU041992	KU042026	KU163351	KU041964
Scolytinae	Hylesinini	*Dactylipalpus grouvellei*	HlDac01	Ghana	HQ883656	HQ883731	HQ883570	HQ883795	HQ883886	–	–	KU042032	–	–
Scolytinae	Hylesinini	*Hylesinus varius*	HlHyl02	Sweden	HQ883657	AF308409	AF308365	HQ883796	HQ883887	KX160760	KX160709	–	KX160647	KX160560
Scolytinae	Hylesinini	*Phloeoborus* sp.	HlPhb02	Guyana	HQ883658	–	HQ883571	HQ883797	HQ883888	–	–	–	–	KU041968
Scolytinae	Hylurgini	*Dendroctonus terebrans*	ToDen02	USA	HQ883680	HQ883749	HQ883591	HQ883824	–	KX160773	–	–	–	–
Scolytinae	Hylurgini	*Tomicus piniperda*	ToTom01	Norway	HQ883681	HQ883750	HQ883592	HQ883825	HQ883911	KX160774	KX160721	–	KX160657	KX160569
Scolytinae	Scolytini	*Camptocerus aenipennis*	ScCam02	Guyana	HQ883676	HQ883745	HQ883587	HQ883818	HQ883907	KX160769	KX160717	KX160637	KX160652	–
Scolytinae	Scolytini	*Cnemonyx vismiaecolens*	ScCne01	Guyana	EU191865	EU191897	HQ883588	HQ883819	HQ883908	KX160770	KX160718	–	KX160653	–
Scolytinae	Scolytini	*Scolytus intricatus*	ScScl02	Sweden	HQ883677	HQ883746	HQ883589	HQ883820	HQ883909	KX160771	KX160719	KX160638	–	–

of invertebrates, but has been briefly considered for such analyses in vertebrates (Fong & Fujita 2011).

Primers and protocols for the five new gene fragments were recently developed for Scolytinae (Table 1; see also Pistone et al. 2016). Five additional markers previously used in weevils were also included (Jordal et al. 2011): arginine kinase (ArgK), carbamoyl-phosphate synthetase 2 - aspartate transcarbamylase - and dihydroorotase (CAD), elongation factor 1 alpha (EF-1α), the large nuclear ribosomal subunit (28S rDNA), and the mitochondrial gene cytochrome oxidase I (COI).

Nucleotide sequences were blasted in GenBank for verification (minimum E value threshold = 1E-4). Orthology was assessed in OrthoDB (Zdobnov et al. 2017) and each of the five novel markers was analysed phylogenetically and compared to the combined result of five established markers (see also Pistone et al. 2016). Sequences were aligned using ClustalX in BioEdit (Hall 1999) and MAFFT (Katoh & Standley 2013) applying default settings. Gblocks (Castresana 2000) was used to reduce the number of ambiguous sites in the 28S rDNA alignment. Settings in Gblocks allowed less strict flanking positions, gap positions within blocks, and small final blocks. In the final matrix, the introns were removed from all the protein coding genes before the phylogenetic analysis.

Phylogenetic analyses were made in a Bayesian statistical framework, or by maximum likelihood, or the principle of parsimony. Four analyses were based on concatenated datasets created for 72 taxa: i) a nucleotide matrix combining 10 gene fragments (5,966 characters) and divided into seven partitions (28S, COI by coding position, and all nuclear coding genes combined by codon position); ii) the same dataset partitioned by 10 genes; iii) including the same 10 genes, but with third codon positions excluded and the remaining data divided into 5 partitions (28S, COI first and second positions, and other protein coding genes first and second positions); iv) a concatenated amino acid matrix (1,792 characters), with nine partitions divided by gene (ribosomal DNA excluded). Additional Bayesian analyses were made of single genes, or combinations of these.

For the Bayesian analyses, MrModeltest v. 2.3 (Posada & Crandall 1998) was used to determine the best substitution model for each partition based on the Akaike information criterion (AIC). The best model for each of the ten genes, and for each codon position, was the general time reversible model with gamma distributed rates and a proportion of invariable sites (GTR+I+Γ). The mixed model was used for amino acid data. The analyses were implemented in MrBayes 3.2.6 (Ronquist & Huelsenbeck 2003), via the CIPRES Science Gateway portal (Miller et al. 2011). Two sets of four Markov Chain Monte Carlo (MCMC) chains (one cold and three heated with default temperature parameter 0.2) were run for 20,000,000 generations (for amino acid data 30,000,000) and sampled every 1,000 generations. The first 25% trees were discarded as burn-in, to obtain a final tree sample of 15,000 trees. Analysis parameters (e.g. likelihood and posterior values) were visualized in the software Tracer1.6 (Rambaut et al. 2014).

Table 3. The number of species successfully sequenced per family or subfamily. Sequencing success higher than 50% is marked in grey.

Family – subfamily	# species	UBA5	PAPB1	Iap2	Arr2	TPI
Anthribidae	3	1	2	3	2	1
Attelabidae	2	0	1	0	1	0
Brentidae / Apionidae	5	3	4	0	2	1
Brachycerinae – Erirhininae	1	0	1	0	0	0
Baridinae – Ceutorhynchinae	2	1	2	2	0	2
Baridinae – Conoderinae	7	4	6	6	4	4
Coptonotinae	2	0	2	0	2	1
Cossoninae	8	7	6	5	4	3
Curculioninae	1	0	1	0	0	0
Dryophthorinae	5	4	4	2	0	3
Entiminae	5	1	3	2	0	0
Molytinae	4	4	4	4	1	3
Molytinae – Cryptorhynchinae	3	2	3	1	1	2
Molytinae – Lixinae	2	2	1	2	1	0
Platypodinae	12	9	11	6	7	6
Scolytinae	10	7	8	5	6	5
Total sequencing success	**72**	**45**	**59**	**38**	**31**	**31**

Maximum likelihood analyses were performed by the software IQTREE (Trifinopoulos et al. 2016). Substitution models for each partition were selected using Model Finder (Kalyaanamoorthy et al. 2017) integrated in the software. Node support was assessed by 1,000 bootstrap replicates.

Parsimony analyses were made in PAUP* (Swofford 2002) with 1,000 random addition replicates and TBR branch swapping. Gaps were treated as missing data and all characters were either equally weighted or third positions were excluded. To assess node support, a total of 200 bootstrap replicates were performed, using 10 random addition replicates per bootstrap replicate.

Phylogenetic trees were visualized in FigTree1.3 (Rambaut & Drummond 2009) and edited in TreeGraph2 (Stover & Muller 2010).

3. Results

A total of 204 sequences were obtained for the five new markers, and 45 additional sequences were obtained for five previously established markers (Tables 2, 3). PABP1 generated readable sequences in 81.9% of the samples, followed by UBA5 (62%), Iap2 (53%), TPI (43%), and Arr2 (43%).

The majority of failures were caused by negative PCR amplification (126 of 146 failures). The most frequent problem with erroneous sequences was either unreadable or chimeric chromatograms (17/146), especially for UBA5, PABP1, and Iap2. Only three sequences of TPI were from other organisms – one nematode and two

Fig. 2. Examples of length variable regions in the Iap2 and Arr2 genes, framed by red boxes. **A**: Iap2 alignments of nucleotides and amino acids (the first of two variable regions). **B**: Arr2 alignments of nucleotides and amino acids.

fungi. Sequences of PABP1 and UBA5 were relatively easy to align, whereas the amplified fragments of Arr2, Iap2 and TPI were more problematic due to long introns (Electronic Supplement Table S1), but also because of one or two length-variable coding regions (Fig. 2). Intron boundaries followed the general GT-AG rule in all genes, except for TPI in two species of *Diapus* Chapuis, 1865 which had the first intron boundaries defined by GC-AG.

3.1. Characteristics of new phylogenetic markers

3.1.1. ***Inhibitor of apoptosis 2* – Iap2.** A total of 38 good quality sequences (clearly defined peaks in the chromatogram) (52.7%) were obtained for this marker. The amplified fragment contained in most cases two exons and one intron (Electronic Supplement Table S1). The intron length varied from 50–274 bp, and was present in most species except Anthribidae, one Molytinae, one Dryophthorinae, and two Platypodinae. The first exon of the amplified gene fragment contained two length-variable regions, resulting in 208–222 amino acids. These length variable regions contained long serine repeats that were difficult to align; hence they were tentatively included or excluded in the phylogenetic analyses.

3.1.2. ***Arrestin2* – Arr2.** Sequences were obtained from 31 of the 72 species (43%). The amplified gene fragment consisted of three exons and two introns. The beginning of the second exon contained indels that translated into a variable number of amino acids (Fig. 2). One triplet insertion occurred in the fourth exon only in *Microborus angustus* Jordal, 2017. The first intron varied from 50–154 bp and was present in the majority of the advanced weevil species with the exclusion of *Coptonotus* and one Conoderinae species, and was absent in Brentidae, Attelabidae, and Anthribidae. The second intron was 49–201 bp long, and occurred in the majority of taxa as described for the first intron. The third intron was 47–84 bp long and present in the majority of the sequences, with the exclusion of 4 species (one Anthribidae, *Mecopelmus zeteki* Blackman, 1944, *M. boops*, and *Hylesinus varius* (Fabricius, 1775).

3.1.3. ***Polyadenylate-binding protein 1* – PABP1.** Sequences were obtained from 59 species (81.9%). The total length was 441 bp, which translates into 147 amino acids. One triplet deletion occurred in *Tesserocerus ericius* Blandford, 1895, resulting in one amino acid shorter fragment. Only one of the 58 successful samples contained an intron (Anthribidae, 156 bp).

Table 4. Posterior probability (pp), parsimony bootstrap support (P-bs) and maximum likelihood bootstrap support (MLbs) from analyses of data set II–IV, for selected groups of weevils supported by data set I (Fig. 3). Bootstrap support values below 50 and posterior probabilities below 0.95 are not shown.

	II: by gene			III: 3rd excluded			IV: amino acids		
	pp	P-bs	MLbs	pp	P-bs	MLbs	pp	P-bs	MLbs
A: Platypodinae + Dryophthorinae	1.0	–	69	1.0	<50	82	<.95	<50	89
B: Dryophthorinae	1.0	71	100	1.0	77	90	1.0	<50	100
C: Platypodinae, excl. *Mecopelmus*	1.0	96	100	1.0	100	100	<.95	86	97
D: Curculionidae, excl. *Mecopelmus*	1.0	–	81	1.0	74	98	<.95	<50	77
E: Curculionidae w/ pedal aedeagus	–	<50	50	–	–	–	<.95	<50	–
F: Entiminae	<.95	56	98	1.0	55	60	<.95	<50	74

3.1.4. ***Ubiquitin-like modifier-activating enzyme 5* – UBA5.** A total of 45 sequences (62%) were obtained for UBA5, consisting of two exons and one intron. The total length of the alignment was 348 bp without the intron. The length of the intron ranged from 50–177 bp, but was absent in *Zacladus* Reitter, 1913, *Microborus,* and one Brentidae. One of the Platypodinae species (*Diapus pusillimus* Chapuis, 1865) had one amino acid insertion in the second exon, resulting in one amino acid longer peptide (116 aa).

3.1.5. ***Triose phosphate isomerase* – TPI.** Among the 39 samples that successfully amplified (54%), only 31 provided validated sequences of sufficient quality (43%). The amplified gene fragment contained up to three introns separating four exons, with 564 bp translated into 188 amino acids. The sequence of *Scolytoproctus* Faust, 1895 (Conoderinae *sensu* Alonso-Zarazaga & Lyal 1999) had one triplet deletion in the fourth exon. The first intron was 50–795 bp long, and was present in most species. The second intron was present only in one species, *Apion curtirostre* Germar, 1817 and was 116 bp long. This additional intron was not observed in our previous screening on bark and ambrosia beetles and other weevils (Pistone et al. 2016). The third intron was 54–246 bp long, and was present in all amplified taxa, except *Z. affinis* (Ceutorhynchinae).

3.2. Phylogenetic analyses

The Bayesian and parsimony analyses of the combined nucleotide data produced largely congruent results for major weevil clades (Table 4). Exclusion or inclusion of the indel-rich coding regions in Arr2 and Iap2 did not change the reconstruction of these clades.

The Bayesian analysis partitioned by codon position per genome and 28S (7 partitions, analysis I) resulted in a paraphyletic Curculionidae with respect to *Mecopelmus zeteki* (Fig. 3). A sister relationship between the core Platypodinae and Dryophthorinae was maximally supported (PP = 1) and these two lineages formed a strongly supported sister group to all other Curculionidae. The Erirhininae (Brachycerinae) genus *Himasthlophallus* Zherikhin & Egorov, 1990 formed a weakly supported sister group to Entiminae and a clade consisting of Scolytinae, Molytinae, Ceutorhynchinae, Cryptorhynchinae, Curculioninae, Cossoninae, Conoderinae, and Lixinae (Baridinae). Each of the last five subfamilies was paraphyletic as defined by Oberprieler et al. (2007), while many smaller clades were consistent with the Alonso-Zarazaga & Lyal (1999) subfamily system. A near-identical topology was found by maximum likelihood using the same 7 partitions in IQTREE (Electronic Supplement Fig. S1).

With the same data partitioned by gene (analysis II), Curculionidae was monophyletic, albeit with *Mecopelmus* and *Apion* Herbst, 1797 forming a basal polytomy (Electronic Supplement Fig. S2). The tree topology was largely congruent with that based on the 7-partitions analysis, but notably with *Himasthlophallus* as sister to Entiminae.

In the parsimony analysis of all nucleotides unweighted, Curculionidae was recovered as paraphyletic with respect to *Apion* and *Apoderus* Olivier, 1897, with bootstrap support of 76 (Electronic Supplement Fig. S2). Excluding the third codon position from the protein coding genes (analysis III) did not result in greater resolution, or higher node support for relationships between subfamilies, in either the Bayesian or the parsimony analyses (Electronic Supplement Fig. S3). A close affinity between the core Platypodinae and Dryophthorinae was again confirmed with maximum support (PP = 1), whereas *Mecopelmus* grouped with *Apion*. In the parsimony analysis with third positions excluded (III), Entiminae was recovered as monophyletic (BS = 55), while in the Bayesian tree, *Sitona* Germar, 1817 (Entiminae) was nested inside Dryophthorinae.

The analysis of the amino acid translated data (analysis IV) resulted in very similar tree topologies in the parsimony and the Bayesian analyses (Electronic Supplement Fig. S4). Both analyses recovered each of the subfamilies Platypodinae (ex *Mecopelmus*) and Dryophthorinae as monophyletic and as sister clades (PP = 0.56); these two groups formed the sister lineage to all other Curculionidae (PP = 0.51). Among the latter, a monophyletic Entiminae (PP = 0.62) formed the sister group to the remaining taxa, but with a marginal posterior probability of 0.56. In the parsimony analysis of these data, *Sitona* (Entiminae) grouped together with parts of Baridinae, and *Mecopelmus* grouped with Entiminae. A moderately supported clade (PP = 0.92) included taxa of Molytinae, Cossoninae, Scolytinae, Curculioninae, Baridinae, and

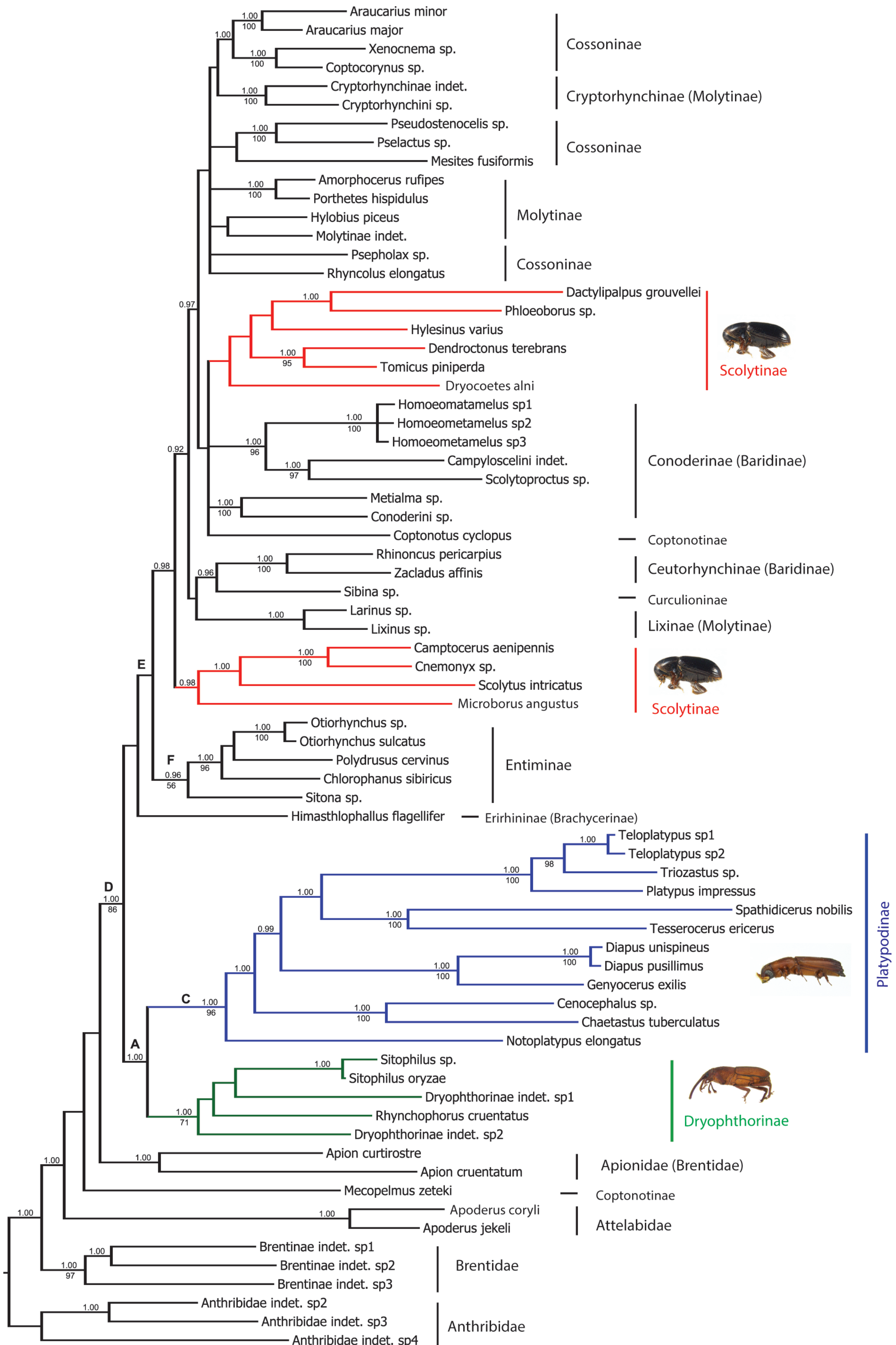

Fig. 3. Phylogenetic consensus tree of dataset I, divided into seven partitions (by codon position in mitochondrial and nuclear genes, and 28S). Bayesian posterior probability values are shown above nodes, and parsimony bootstrap values below nodes.

Coptonotus, forming largely a polytomy. Conoderinae was monophyletic (PP = 0.79) and was closely related to the molytine tribe Amorphocerini (PP = 0.54). In both the Bayesian and parsimony analyses, *Coptonotus* grouped together with part of a paraphyletic Scolytinae.

Separate analyses of the five established markers combined, and the five new markers combined, resulted in less resolved tree topologies compared to the analyses of all data (Electronic Supplement Fig. S5). In each case Curculionidae was monophyletic. The most significant difference between the two smaller datasets was a sister relationship between Platypodinae and Dryophthorinae that was supported by the new markers only (PP = 0.95). In the nucleotide analyses of individual genes (Electronic Supplement Figs. S6–S9), the Platypodinae-Dryophthorinae clade was supported by the ArgK and UBA5 data, and nearly so by the TPI data. All Dryophthorinae were lacking Arr2 data, while Iap2 indicated a more derived position for Dryophthorinae, separate from Platypodinae. Amino acid translated data revealed largely paraphyletic groups for most of the genes, except COI, Arr2, TPI, and Iap2, which were all monophyletic for Platypodinae, whereas TPI grouped Platypodinae and Dryophthorinae as sister groups (Electronic Supplement Fig. S7).

4. Discussion

4.1. Weevil relationships

This study provides the clearest evidence to date for a sister relationship between Dryophthorinae and the core Platypodinae (*sensu* JORDAL 2015). Previous molecular studies have suggested similar topologies, but these had generally lower node support, including for this particular node (MCKENNA et al. 2009; GILLETT et al. 2014). With maximum support in the various analyses presented here, it seems prudent to conclude that these two subfamilies are indeed sister groups. Our molecular data therefore refute a close relationship between Scolytinae and Platypodinae which has been proposed repeatedly over the last centuries (BLANDFORD 1897; SCHEDL 1972; WOOD 1978; KUSCHEL 1995; KUSCHEL et al. 2000; BRIGHT 2014), even in mixed molecular- and morphology-based analyses (MARVALDI et al. 2002; JORDAL et al. 2011).

Previous comparative analyses of morphological data focussed to a large extent on adult head structures (WOOD 1978, 1986; MORIMOTO & KOJIMA 2003), features that are heavily modified through adaptation to wood boring and therefore not necessarily homologous in taxa with similar feeding behaviour (e.g. LYAL 1995). Several other features associated with wood tunnelling show extensive homoplasy, including the shape of legs with hooks and denticles used for substrate attachment, for instance in unrelated groups such as Campyloscelini (Conoderinae) and in Araucariini (Cossoninae, see e.g. KUSCHEL 1966; JORDAL et al. 2011). Larval anatomy, which may be less prone to wood boring adaptations, supports a sister relationship between Platypodinae and Dryophthorinae at the base of Curculionidae (MARVALDI 1997). A more detailed review of the historical development of morphology-based classifications of Platypodinae and Scolytinae can be found in JORDAL (2014).

Our molecular data corroborate recent studies that excluded *Mecopelmus* from Platypodinae, supporting a more narrowly defined subfamily that corresponds to the core Platypodidae *sensu* WOOD (1993) or Platypodinae *sensu* JORDAL (2015). This is generally consistent with morphological characters, in particular the male genitalia and associated abdominal structures, which are very different in *Mecopelmus* (see THOMPSON 1992; KUSCHEL et al. 2000; JORDAL 2014). Larvae are unfortunately not known for this genus, which could potentially have clarified the relationship to other weevil groups. The position of *Mecopelmus* therefore appears to be one of the major remaining challenges in weevil phylogenetics, and requires considerably more sequence data to solve.

Several molecular studies have indicated that Platypodinae and Dryophthorinae are, together with members of the Brachycerinae, distinct basal lineages in Curculionidae (MCKENNA et al. 2009; GILLETT et al. 2014). The split between these three groups and the remaining Curculionidae (including Entiminae) is supported by major differences in the male genitalia – with Entiminae and other derived Curculionidae having a pedal form as opposed to the ancestral pedotectal type seen in Dryophthorinae and Brachycerinae (THOMPSON 1992). The male genitalia of Platypodinae are highly reduced and therefore difficult to assess, but they have tentatively been associated with the more primitive type of genitalia. Molecular data strongly support the assertion that the platypodine aedeagus is derived from the pedotectal type. Brachycerinae, Dryophthorinae and Platypodinae are ranked as subfamilies in the OBERPRIELER et al. system (2007), while given full family status in the ALONSO-ZARAZAGA & LYAL system (1999). In light of the recent phylogenetic results, it is understandable that such discrepancies in rank occur. Without defined auxiliary criteria, such as the time banding criterion (VENCES et al. 2013), the rank seems largely subjective. A reconciled solution would need additional information on the Brachycerinae in particular, a group which may consist of multiple unrelated lineages (MCKENNA et al. 2009; GILLETT et al. 2014) and, hence, will be simultaneously affected by changes in the rank of Dryophthorinae and Platypodinae (see also JORDAL et al. 2014).

Our study also confirms a long-standing hypothesis that Entiminae form part of a distinct lineage of broad-nosed weevils placed among the more advanced Curculionidae. Data on mitochondrial genomes have also shown that Cyclominae and Hyperinae (*sensu* ALONSO-ZARAZAGA & LYAL 2009) belong to this lineage (GILLETT et al. 2014; GUNTER et al. 2015). Together they form the sister group to all other advanced weevils, including Cossoninae, Scolytinae, a broadly defined Molytinae, Curculioninae, and Baridinae (see also MCKENNA et al. 2009).

The advanced weevil clade also includes the genus *Coptonotus*, which therefore has a very distant relationship to *Mecopelmus* – both of which have been placed in the same family Coptonotinae by some authors (e.g. Schedl 1962; Wood 1993; Wood & Bright 1992). Molecular data were indecisive in placing *Coptonotus* which seems to be an old isolated lineage consisting of only four known species (Smith & Cognato 2016).

The limited resolution of the major lineages of advanced weevils is not very surprising given the enormous diversity characterising this part of the weevil tree. Relationships among Curculioninae, Molytinae, and Baridinae (*sensu* Oberprieler et al. 2007) were largely unresolved also in previous molecular studies, including those based on mitochondrial genomes (Haran et al. 2013; Gillett et al. 2014). Most of the incongruence found in our study is mainly associated with the deepest nodes in each of these subfamilies, reflecting potential problems with the broad concept of classification proposed by Oberprieler et al. (2007). The Alonso-Zarazaga & Lyal (1999) system is on the other hand more finely divided into many more subfamilies and each of these is therefore less likely to be polyphyletic. Consistent with the latter system we recovered separate clades for the 'baridine' groups Ceutorhynchinae and Conoderinae, and separate clades for the 'molytine' groups Lixinae, Cryptorhynchinae, and Molytinae *sensu stricto*. However, our taxonomic sampling was limited to just a few genera for each of these groups and can therefore not provide a proper test of monophyly. A recent molecular study on Cryptorhynchinae illustrated, for instance, the many problems with placing atypical members of 'molytine' subgroups (Riedel et al. 2016).

4.2. Application of novel molecular markers

The optimization and application of five new molecular markers in weevil phylogenetics was promising despite a variable degree of PCR amplification. A modest increase in new molecular data – less than doubling the number of nucleotides – gave increased node support for the Dryophthorinae-Platypodinae clade in particular, but also in the node connecting Scolytinae, Cossoninae, Curculioninae, and the broadly defined Baridinae and Molytinae (compared to McKenna et al. 2009; Gillett et al. 2014). Several deeper nodes on the other hand appeared to conflict with well-established topologies, indicating high substitution rates in many of these markers. They therefore seem to have limited potential in resolving older weevil relationships (see Pistone et al. 2016). Moreover, we obtained low resolution in the most diverse clade of Curculionidae, similar to recent phylogenetic studies based on complete or partial mitochondrial genomes (Haran et al. 2013; Gillett et al. 2014; Gunter et al. 2015). In general, it appears difficult to obtain resolution in this most diverse section of the weevil tree, and is likely a consequence of high diversity, involving tens of thousands of species (Oberprieler et al. 2007).

Low resolution could also be due to missing data, particularly in TPI, Arr2, and Iap2, which were problematic to amplify across all Curculionoidea. These gene fragments sometimes contained very long introns that may require further optimization of PCR extension times and improved primer design. Furthermore, some primers appear to be taxon specific, such as Iap2, which mainly amplified species of Anthribidae, Molytinae, Baridinae, and Cossoninae; TPI, which mainly amplified species of Molytinae, Baridinae and Dryophthorinae; while the Arr2 and TPI primers did not amplify any Entiminae. The same three genes were also problematic to align, in part due to the irregular length of introns, and in Iap2 and Arr2 this was also due to length variable coding regions. These length variable regions may be informative for certain clades (Pistone et al. 2018), but their signature varies considerably among weevil taxa and is generally known to be rather homoplasious across families and orders of insects (Ajawatanawong & Baldauf 2013; Hardy 2007).

Incongruence of single genes may also contribute to reduced resolution in the weevil tree topology. The single most deviant gene in this respect was Iap2, which placed Dryophthorinae in a highly supported derived position separate from Platypodinae. However, this strong support faded when the data were translated to amino acids and became more similar in topology to the TPI data. There is a slight possibility that some of the genes include a mixture of multiple gene copies, which is known for some genes such as Elongation Factor 1-alpha in bark beetles (Jordal 2002). Different copies of this gene can nonetheless be detected by different intron structure and highly divergent sequences, but were not observed in our dataset. Among the other 9 genes we could not detect any signs of paralogous copies based on OrthoDB analyses using all available Coleoptera and Hymenoptera sequences. It is therefore not very likely that paralogous copies are responsible for the observed incongruence across individual genes. Rather, it is anticipated that single genes are not able to provide phylogenetic signals that correspond to comprehensive multi-gene analyses (McKenna et al. 2009; Gillet et al. 2014). Instead, we observed a significant increase in resolution and node support with a stepwise addition of five new markers. The clearest indication of such accumulative effects from the new data was the better resolution of the core Platypodinae, which was monophyletic or nearly so for Arr2, UBA5, Iap2, and TPI, while only 28S among the established markers supported monophyly of the subfamily.

To enable a more complete resolution in the phylogeny of main weevil groups, larger volumes of genomic data are required. New data are currently being processed as a part of the 1-Kite project where a broadly sampled weevil phylogeny will be reconstructed from more than 1,000 loci (McKenna et al. unpubl. data) obtained by anchored hybrid enriched sequence capture (Lemmon et al. 2012). This approach will likely become the standard procedure in large scale phylogenetics in the future, which could make PCR-based Sanger sequencing redundant (Brady et al. 2014; Faircloth et al. 2015). Howev-

er, most phylogenies made in connection with taxonomic work are more practically obtained with smaller data volumes. Given that PCR amplification of few genes and individuals is still much faster and cheaper than next generation sequencing, the Sanger method will still be needed for small-scale routine phylogenetics such as DNA barcoding and integrative taxonomy. Thus, our twofold aim here was to develop primers and protocols for new molecular markers, and to use the new data to test one particularly interesting relationship – the one between Dryophthorinae and Platypodinae. We believe the new data obtained have demonstrated considerable promise in achieving these aims.

5. Acknowledgements

We would like to thank Jiri Hulcr and Douglas Downie for important samples. This research was funded by the Norwegian Research Council, grant 214232/F20.

6. References

Ajawatanawong P., Baldauf S.L. 2013. Evolution of protein indels in plants, animals and fungi. – BMC Evolutionary Biology **13**: 140.

Alonso-Zarazaga M.A., Lyal C.H.C. 1999. A world catalogue of families and genera of Curculionoidea (Insecta: Coleoptera) (excepting Scolytidae and Platypodidae). – Entomopraxis. S.C.P. Edition, 315 pp.

Apponi L.H., Leung S.W., Williams K.R., Valentini S.R., Corbett A.H., Pavlath G.K. 2010. Loss of nuclear poly(a)-binding protein 1 causes defects in myogenesis and mRNA biogenesis. – Human Molecular Genetics **19**: 1058–1065.

Bacik J.-P., Walker J.R., Ali M., Schimmer A.D., Dhe-Paganon S. 2010. Crystal structure of the human ubiquitin-activating enzyme 5 (uba5) bound to ATP: Mechanistic insights into a minimalistic e1 enzyme. – Journal of Biological Chemistry **285**: 20273–20280.

Beaver R.A. 1989. Insect-fungus relationships in the bark and ambrosia beetles. Pp. 121–143 in: Wilding N.M.C.N., Hammond P.M., Webber J.F. (eds), Insect-fungus Interactions. – Academic Press, London.

Blandford W.F.H. 1897. Family Scolytidae. Pp. 145–184 in: Biologia Centrali-Americana, Coleoptera.

Brady S.G., Fisher B.L., Schultz T.R., Ward P.S. 2014. The rise of army ants and their relatives: Diversification of specialized predatory doryline ants. – BMC Evolutionary Biology **14**: 93.

Bright D.E. 2014. A catalog of Scolytidae and Platypodidae (Coleoptera), supplement 3 (2000–2010), with notes on subfamily and tribal reclassifications. – Insecta Mundi **356**: 1–336.

Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. – Molecular Biology and Evolution **17**: 540–552.

Chang J.C., Ramasamy S. 2013. Molecular-phylogenetic characterization of arrestin-2 from maruca vitrata (Lepidoptera: Crambidae). – Annals of the Entomological Society of America **106**: 359–370.

Dou T., Gu S., Liu J., Chen F., Zeng L., Guo L. et al. 2005. Isolation and characterization of ubiquitin-activating enzyme e1-domain containing 1, ube1dc1. – Molecular Biology Reports **32**: 265–271.

Faircloth B.C., Branstetter M.G., White N.D., Brady S.G. 2015. Target enrichment of ultraconserved elements from arthropods provides a genomic perspective on relationships among Hymenoptera. – Molecular Ecology Resources **15**: 489–501.

Farrell B.D. 1998. “Inordinate fondness” explained: Why are there so many beetles? – Science **281**: 555–559.

Farrell B.D., Sequeira A.S., O’Meara B.C., Normark B.B., Chung J.H., Jordal B.H. 2001. The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). – Evolution **55**: 2011–2027.

Fong J.J., Fujita M.K. 2011. Evaluating phylogenetic informativeness and data-type usage for new protein-coding genes across Vertebrata. – Molecular Phylogenetics and Evolution **61**: 300–307.

Gavin J.M., Hoar K., Xu Q., Ma J., Lin Y., Chen J. et al. 2014. Mechanistic study of Uba5 enzyme and the Ufm1 conjugation pathway. – Journal of Biological Chemistry **289**: 22648–22658.

Gillett C.P.D.T., Crampton-Platt A., Timmermans M.J.T.N., Jordal B.H., Emerson B.C., Vogler A.P. 2014. Bulk de novo mitogenome assembly from pooled total DNA elucidates the phylogeny of weevils (Coleoptera: Curculionoidea). – Molecular Biology and Evolution **31**: 2223–2237.

Gunter N., Oberprieler R., Cameron S. 2015. Molecular phylogenetics of Australian weevils (Coleoptera: Curculionoidea): Exploring relationships in a hyperdiverse lineage through comparison of independent analyses. – Austral Entomology **55**: 217–233.

Gurevich E.V., Gurevich V.V. 2006. Arrestins: Ubiquitous regulators of cellular signaling pathways. – Genome Biology **7**: 236.

Hall T.A. 1999. Bioedit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/nt. – Nucleic Acids Symposium Series **41**: 95–98.

Haran J., Timmermans M.J.T.N., Vogler A.P. 2013. Mitogenome sequences stabilize the phylogenetics of weevils (Curculionoidea) and establish the monophyly of larval ectophagy. – Molecular Phylogenetics and Evolution **67**: 156–166.

Hardy N.B. 2007. Phylogenetic utility of dynamin and triose phosphate isomerase. – Systematic Entomology **32**: 396–403.

Huh J.R., Foe I., Muro I., Chen C.H., Seol J.H., Yoo S.J. et al. 2007. The drosophila inhibitor of apoptosis (IAP) DIAP2 is dispensable for cell survival, required for the innate immune response to gram-negative bacterial infection, and can be negatively regulated by the reaper/hid/grim family of IAP-binding apoptosis inducers. – Journal of Biological Chemistry **282**: 2056–2068.

Hulcr J., Atkinson T.H., Cognato A.I., Jordal B.H., McKenna D.D. 2015. Morphology, taxonomy, and phylogenetics of bark beetles. Pp. 41–84 in: Vega F.E., Hofstetter R.W. (eds), Bark Beetles – Biology and Ecology of Native and Invasive Species. – Academic Press, London.

Jordal B.H. 2002. Elongation Factor 1α resolves the monophyly of the haplodiploid ambrosia beetles Xyleborini (Coleoptera: Curculionidae). – Insect Molecular Biology **11**: 453–465.

Jordal B.H. 2014. Platypodinae. Pp. 358–364 in: Leschen R.A.B., Beutel R. (eds), Handbook of Zoology: Coleoptera Beetles. Volume 3: Morphology and Systematics. – De Gruyter Press, Berlin.

Jordal B.H. 2015. Molecular phylogeny and biogeography of the weevil subfamily Platypodinae reveals evolutionarily conserved range patterns. – Molecular Phylogenetics and Evolution **92**: 294–307.

Jordal B.H., Cognato A.I. 2012. Molecular phylogeny of bark and ambrosia beetles reveals multiple origins of fungus farming during periods of global warming. – BMC Evolutionary Biology **12**: 133.

Jordal B.H., Sequeira A.S., Cognato A.I. 2011. The age and phylogeny of wood boring weevils and the origin of subsociality. – Molecular Phylogenetics and Evolution **59**: 708–724.

Jordal B.H., Smith S.M., Cognato A.I. 2014. Classification of weevils as a data-driven science: Leaving opinion behind. – ZooKeys **439**: 1–18.

Katoh K., Standley D.M. 2013. Mafft multiple sequence alignment software version 7: Improvements in performance and usability. – Molecular Biology and Evolution **30**: 772–780.

Kirkendall L.R., Biedermann P.H.W., Jordal B.H. 2015. Diversity and evolution of bark beetles. Pp. 85–156 in: Vega F.E., Hofstetter R.W. (eds), Bark Beetles – Biology and Ecology of Native and Invasive Species. – Academic Press, London.

Komatsu M., Chiba T., Tatsumi K., Iemura S., Tanida I., Okazaki N. et al. 2004. A novel protein-conjugating system for Ufm1, a ubiquitin-fold modifier. – EMBO Journal **23**: 1977–1986.

Kuschel G. 1966. A cossonine genus with bark-beetle habits, with remarks on relationships and biogeography (Coleoptera Curculionidae). – New Zealand Journal of Science **9**: 3–29.

Kuschel G. 1995. A phylogenetic classification of Curculionoidea to families and subfamilies. – Memoirs of the Entomological Society of Washington **14**: 5–33.

Kuschel G., Leschen R.A.B., Zimmerman E.C. 2000. Platypodidae under scrutiny. – Invertebrate Taxonomy **14**: 771–805.

Lemmon A.R., Emme S.A., Lemmon E.M. 2012. Anchored hybrid enrichment for massively high-throughput phylogenomics. – Systematic Biology **61**: 727–744.

Leulier F., Ribeiro P.S., Palmer E., Tenev T., Takahashi K., Robertson D. et al. 2006. Systematic in vivo RNAi analysis of putative components of the drosophila cell death machinery. – Cell Death and Differentiation **13**: 1663–1674.

Lyal C.H.C. 1995. The ventral structures of the weevil head (Coleoptera: Curculionoidea). – Memoirs of the Entomological Society of Washington **14**: 35–51.

Marvaldi A.E. 1997. Higher level phylogeny of Curculionidae (Coleoptera: Curculionoidea) based mainly on larval characters, with special reference to broad-nosed weevils. – Cladistics **13**: 285–312.

Marvaldi A.E., Sequeira A.S., O'Brien C.W., Farrell B.D. 2002. Molecular and morphological phylogenetics of weevils (Coleoptera, Curculionoidea): Do niche shifts accompany diversification? – Systematic Biology **51**: 761–785.

McKenna D.D., Farrell B.D. 2010. 9-genes reinforce the phylogeny of Holometabola and yield alternate views on the phylogenetic placement of Strepsiptera. – PLoS ONE **5**: e11887.

McKenna D.D., Sequeira A.S., Marvaldi A.E., Farrell B.D. 2009. Temporal lags and overlap in the diversification of weevils and flowering plants. – Proceedings of the National Academy of Science USA **106**: 7083–7088.

Miller M.A., Pfeiffer W., Schwartz T. 2011. The CIPRES Science Gateway: A community resource for phylogenetic analyses. Pp. 1–8 in: Proceedings of the 2011 TeraGrid Conference: Extreme Digital Discovery. – ACM, Salt Lake City, Utah.

Morimoto K., Kojima H. 2003. Morphologic characters of the weevil head and phylogenetic implications (Coleoptera, Curculionoidea). – Esakia: occasional papers of the Hikosan Biological Laboratory in Entomology **43**: 133–169.

Mueller U.G., Gerardo N. 2002. Fungus-farming insects: Multiple origins and diverse evolutionary histories. – Proceedings of the National Academy of Science USA **99**: 15247–15249.

Oberprieler R.G., Anderson R.S., Marvaldi A.E. 2014. Curculionoidea Latreille, 1802: Introduction, phylogeny. Pp. 285–300 in: Leschen R.A.B., Beutel R. (eds), Handbook of Zoology: Coleoptera Beetles. Volume 3: Morphology and Systematics. – De Gruyter Press, Berlin.

Oberprieler R.G., Marvaldi A.E., Anderson R.S. 2007. Weevils, weevils, weevils everywhere. – Zootaxa **1668**: 491–520.

Pistone D., Mugu S., Jordal B.H. 2016. Genomic mining of phylogenetically informative nuclear markers in bark and ambrosia beetles. – PLoS ONE **11**: e0163529. doi:10.1371/journal.pone.0163529.

Pistone D., Gohli J., Jordal B.H. 2018. Molecular phylogeny of bark and ambrosia beetles (Curculionidae: Scolytinae) based on 18 molecular markers. – Systematic Entomology **43**: 387–406.

Posada D., Crandall K.A. 1998. Modeltest: Testing the model of DNA substitution. – Bioinformatics **14**: 817–818.

Rajalingam K., Sharma M., Paland N., Hurwitz R., Thieck O., Oswald M., Machuy N., Rudel T. 2006. IAP-IAP complexes required for apoptosis resistance of *C. trachomatis*-infected cells. – PLoS Pathogens **2**: e114.

Rambaut A., Suchard M.A., Xie D., Drummond A.J. 2014. Tracer v1.6. – Available from http://beast.bio.ed.ac.uk/Tracer.

Rambaut A., Drummond A.J. 2009. Figtree v1. 3.1. – Available from http://tree.bio.ed.ac.uk/software/figtree.

Riedel A., Tänzler R., Pons J., Suhardjono Y.R., Balke M. 2016. Large-scale molecular phylogeny of Cryptorhynchinae (Coleoptera, Curculionidae) from multiple genes suggests American origin and later Australian radiation. – Systematic Entomology **41**: 492–503.

Ronquist F., Huelsenbeck J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. – Bioinformatics **19**: 1572–1574.

Schedl K.E. 1962. Fam. Coptonotidae, Coleoptera. – Genera Insectorum **215**: 1–13.

Schedl K.E. 1972. Monographie der Familie Platypodidae Coleoptera. – W. Junk, Den Haag.

Smith R.W., Blee T.K., Gray N.K. 2014. Poly(a)-binding proteins are required for diverse biological processes in metazoans. – Biochemical Society Transactions **42**: 1229–1237.

Smith S.M., Cognato A.I. 2016. A revision of *Coptonotus* Chapuis, 1869 (Coleoptera: Curculionidae: Coptonotinae) with notes on its biology. – Coleopterists Bulletin **70**: 409–428.

Stover B., Muller K. 2010. Treegraph 2: Combining and visualizing evidence from different phylogenetic analyses. – BMC Bioinformatics **11**: 7.

Swofford D.L. 2002. Paup*: Phylogenetic analysis using parsimony, version 4.0b10. – Sunderland, Massachusetts: Sinauer Associates.

Thompson R.T. 1992. Observations on the morphology and classification of weevils (Coleoptera, Curculionoidea) with a key to major groups. – Journal of Natural History **26**: 835–891.

Vences M., Guayasamin J.M., Miralles A., De la Riva I. 2013. To name or not to name: Criteria to promote economy of change in Linnaean classification schemes. – Zootaxa **3636**: 201–244.

Wiegmann B.M., Trautwein M.D., Kim J.W., Cassel B.K., Bertone M.A., Winterton S.L. et al. 2009. Single-copy nuclear genes resolve the phylogeny of the holometabolous insects. – BMC Biology **7**: 34.

Wierenga R.K., Kapetaniou E.G., Venkatesan R. 2010. Triosephosphate isomerase: A highly evolved biocatalyst. – Cell Molecular Life Science **67**: 3961–3982.

Wood S.L. 1978. A reclassification of the subfamilies and tribes of Scolytidae (Coleoptera). – Annales de Societé Entomologique de France **14**: 95–122.

Wood S.L. 1986. A Reclassification of the Genera of Scolytidae (Coleoptera). – Brigham Young University Press, Provo.

Wood S.L. 1993. Revision of the genera of Platypodidae (Coleoptera). – Great Basin Naturalist **53**: 259–281.

Wood S.L., Bright D. 1992. A catalog of Scolytidae and Platypodidae (Coleoptera). Part 2: Taxonomic index. – Great Basin Naturalist Memoirs **13**: 1–1553.

Zimmerman E.C. 1993. Australian Weevils (Coleoptera: Curculionoidea). Vol. 3: Nanophyidae, Rhynchophoridae, Erirhinidae, Curculionidae: Amycterinae. – CSIRO Press, Sydney.

Zimmerman E.C. 1994. Australian Weevils (Coleoptera: Curculionoidea). Vol. 1: Orthoceri; Anthribidae to Attelabidae, the Primitive Weevils. – CSIRO Press, Sydney.

Zdobnov E.M., Tegenfeldt F., Kuznetsov D., Waterhouse R.M., Simão F.A., Ioannidis P., Kriventseva E.V. 2017. OrthoDB v9.1: cataloging evolutionary and functional annotations for animal, fungal, plant, archaeal, bacterial and viral orthologs. – Nucleic Acids Research **45**: D744–D749. http://doi.org/10.1093/nar/gkw1119.

Electronic Supplement Files

at http://www.senckenberg.de/arthropod-systematics

File 1: mugu&al-curculionidaephylogeny-asp2018-electronicsupplement-1.pdf — **Fig. S1**. Phylogeny resulting from the maximum likelihood analysis I in IQTree, divided into seven partitions (by codon position in mitochondrial and nuclear genes, and 28S). Bootstrap support values are shown on nodes. — **Fig. S2**. Phylogeny resulting from the Bayesian analysis of dataset II, divided into ten partitions (by gene). Posterior probability values above the nodes, and parsimony bootstrap values below. — **Fig. S3.** Phylogeny resulting from the Bayesian analysis of dataset III, divided into five partitions (by 28S, and genome and codon positions with third positions excluded). Posterior probability values above the nodes, parsimony bootstrap values below. — **Fig. S4.** Phylogeny resulting from the Bayesian analysis of amino acid data (dataset IV), divided into nine partition by gene. Posterior probability values above the nodes, parsimony bootstrap values below. — **Fig. S5.** Combined analysis of five gene fragments. **A**: Phylogeny based on Bayesian analysis of EF-1α, CO1, 28S, CAD and ArgK. **B**: Based on TPI, UBA5, Arr2, Iap2 and PABP1. — **Fig. S6.** Tree topologies resulting from the individual Bayesian analyses of PABP1, UBA5, Arr2, Iap2 and TPI. — **Fig. S7.** Tree topologies resulting from the individual Bayesian analyses of amino acid translated data from PABP1, UBA5, Arr2, Iap2 and TPI. — **Fig. S8.** Tree topologies resulting from the individual Bayesian analyses of COI, EF-1α, CAD, 28S and ArgK. — **Fig. S9.** Tree topologies resulting from the individual Bayesian analyses of amino acid translated COI, EF-1α, CAD and ArgK.

File 2: mugu&al-curculionidaephylogeny-asp2018-electronicsupplement-2.doc — **Table S1**. Description of the length of the coding sequence in terms of translated amino acids (aa) and the number of intervening introns. Voucher codes refer to taxa listed in Table 2.