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

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#### 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 ( $28 \mathrm{~S}, \mathrm{COI}, \mathrm{CAD}, \mathrm{ArgK}$, and EF-1 $\alpha$ ). 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 18 S 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 fungusfarming 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 \& Kолima 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 Тномрson 1992; Zimmerman 1993, 1994; Alonso-Zarazaga \& Lyal 1999).

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

| Gene | Primer se | quence | Annealing temperature |
| :---: | :---: | :---: | :---: |
| Large ribosomal subunit (28S) | $\begin{aligned} & \text { (S3690) } \\ & \text { (A4285) } \\ & \text { (A4394) } \end{aligned}$ | GAGAGTTMAASAGTACGTGAAAC CTGACTTCGTCCTGACCAGGC TCGGAAGGAACCAGCTACTA | $55^{\circ} \mathrm{C}(45 \mathrm{~s})$ |
| Arginine kinase (ArgK) | (forB2) (revB1) (LTrev2) | GAYTCCGGWATYGGWATCTAYGCTCC TCNGTRAGRCCCATWCGTCTC GATKCCATCRTDCATYTCCTTSACRGC | $50^{\circ} \mathrm{C}(45 \mathrm{~s})$ |
| Arrestin2 (Arr2) | (F) <br> (R) | CGYGARGAGGAYGARGTYATGGG ACCATSGTRACYTCGCAATGYTGCAC | $52^{\circ} \mathrm{C}(30 \mathrm{~s})$ |
| Carbamoyl-phosphate synthetase 2 (CAD) | (forB2) (for4) (rev1mod) | GARAARGTNGCNCCNAGTATGGC TGGAARGARGTBGARTACGARGTGGTYCG GCCATYRCYTCBCCYACRCTYTTCAT | $50^{\circ} \mathrm{C}(45 \mathrm{~s})$ |
| Cytochrome oxidase I (COI) | $\begin{aligned} & \text { (S1718) } \\ & \text { (A2237) } \\ & \text { (A2411) } \end{aligned}$ | GGAGGATTTGGAAATTGATTAGTTCC CCGAATGCTTCTTTTTTACCTCTTTCTTG GCTAATCATCTAAAAACTTTAATTCCWGTWG | $46^{\circ} \mathrm{C}$ (45s) |
| Elongation factor 1 alpha (EF-1a) | (S149) <br> (A1043) <br> (A754) | ATCGAGAAGTTCGAGAAGGAGGCYCARGAAATGGG GTATATCCATTGGAAATTTGACCNGGRTGRTT CCACCAATTTTGTAGACATC | $58-44^{\circ}{ }^{*}$ |
| Inhibitor of apoptosis 2 (lap2) | (F2) <br> (R) | CCATCKGGCRTGYTCYGTCCAWGGATC TGGAAYTAYGGRGACCAAGTRATGGC | $52^{\circ} \mathrm{C}$ (30s) |
| Polyadenylate binding protein (PABP1) | (F) <br> (R) | CCRATTCGYATYATGTGGTC GAARGCRACAAAWCCRAAWCC | $50^{\circ} \mathrm{C}$ (30s) |
| Triosephosphate isomerase (TPI) | $\begin{aligned} & \text { (46F) } \\ & (615 R) \end{aligned}$ | GGTGGHAACTGGAARATGAACGG CKGARCCYCCRTATTGRATTC | $50^{\circ} \mathrm{C}(45 \mathrm{~s})$ |
| Ubiquitin-like modifier activating enzyme 5 (UBA5) | (F) <br> (R) | TTGGKAGYGTAACWGCRGAAATG ATATGGCCWGARACSGCRTTTTC | $48^{\circ} \mathrm{C}$ (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), Ubiquitinlike modifier-activating enzyme 5 (UBA5), and Polyade-nylate-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

| FAMILY | TRIBE | SPECIES | CODE | COUNTRY | C01 | EF-1 $\alpha$ | 28S | CAD | ArgK | PABP1 | UBA5 | lap2 | Arrestin2 | TPI |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Anthribidae | indet. | Anthribidae indet. sp2 | Antrib02 | Cameroon | H0883608 | H0883696 | - | H0883765 | H0883841 | 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 | H0883609 | - | H0883528 | - | H0883842 | KU041909 | - | - | - | - |
| Attelabidae | Apoderini | Apoderus jekeli | AtApo02 | Russia | H0883610 | H0883697 | H0883529 | - | H0883843 | - | - | - | KU163339 | - |
| Apionidae | Apionini | Apion (Apion) cruentatum | BrApi03 | Norway | KU041890 | KU041898 | KU041867 | - | - | KU041911 | - | - | - | - |
| Apionidae | Apionini | Apion (Perapion) curtirostre | BrApi01 | Norway | H0883612 | H0883698 | H0883531 | H0883767 | - | KU041910 | - | - | - | KU041952 |
| Brentidae | (Brentinae) | Brentinae indet. sp1 | BrBre01 | Sarawak | H0883613 | H0883699 | H0883532 | - | H0883845 | KU041912 | KU041976 | - | - | - |
| Brentidae | (Brentinae) | Brentinae indet. sp2 | BrBre02 | Cameroon | H0883614 | H0883700 | H0883533 | H0883768 | H0883846 | - | KU041977 | - | KU163340 | KU041953 |
| Brentidae | (Brentinae) | Brentinae indet. sp3 | BrBre03 | Cameroon | KU041891 | - | KU041868 | - | KU041878 | KX160751 | KX160704 | - | KX160641 | - |
| Curculionidae |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| SUBFAMILY |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Ceutorhynchinae | Ceutorhynchini | Zacladus affinis | CeZac01 | Norway | H0883621 | H0883706 | H0883540 | H0883772 | H0883853 | KU041917 | KU041980 | KU042016 | - | KU041959 |
| Ceutorhynchinae | Phytobiini | Rhinoncus pericarpius | CeRhi01 | Norway | H0883620 | - | H0883539 | H0883771 | H0883852 | KU041916 | - | KU042015 | - | KU041958 |
| Conoderinae | Campyloscelini | Campyloscelini indet. | CdCod02 | Cameroon | H0883616 | H0883702 | H0883535 | H0883769 | H0883848 | - | KU041978 | KU042012 | - | KU041954 |
| Conoderinae | Campyloscelini | Homoeometamelus sp1 | CsXxA01 | Uganda | H0883643 | H0883723 | H0883558 | H0883785 | H0883872 | 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 | H0883618 | H0883704 | H0883537 | H0883770 | H0883850 | KU041914 | - | KU042013 | - | KU041956 |
| Conoderinae | Conoderini | Conoderini sp. | CdZyg01 | Russia | H0883619 | H0883705 | H0883538 | - | H0883851 | KU041915 | KU041979 | KU042014 | KU163342 | KU041957 |
| Conoderinae | Conoderini | Metialma sp. | CdMet | South Africa | H0883617 | H0883703 | H0883536 | - | H0883849 | KU041913 | - | - | KU163341 | KU041955 |
| Coptonotinae | Coptonotini | Coptonotus cyclopus | CpCop01 | CR | H0883624 | - | - | H0883774 | H0883856 | KU041918 | - | - | KU163344 | KU041960 |
| Coptonotinae | Mecopelmini | Mecopelmus zeteki | MeMec01 | Panama | H0883663 | H0883735 | H0883574 | H0883802 | H0883892 | KU041939 | - | - | KU163353 | - |
| Cossoninae | Araucariini | Araucarius major | CsAru02 | Argentina | AY040285 | H0883711 | AF308350 | - | H0883860 | KU041922 | KU041985 | KU042020 | KU163347 | - |
| Cossoninae | Araucariini | Araucarius minor | CsAru01 | Argentina | AF375307 | AF308346 | AF308351 | - | H0883859 | - | KU041984 | KU042019 | KU163346 | - |
| Cossoninae | Araucariini | Coptocorynus sp. | CsCpt02 | PNG | H0883631 | H0883714 | H0883546 | H0883776 | H0883862 | KU041923 | KU041986 | - | - | KU041963 |
| Cossoninae | Araucariini | Xenocnema sp. | CsXen01 | Australia | H0883642 | H0883722 | H0883557 | - | - | KU041925 | KU041989 | - | - | - |
| Cossoninae | Cossonini | Mesites fusiformis | CsMes01 | Spain | EU191838 | EU191870 | H0883549 | H0883778 | H0883865 | KX160754 | - | - | - | KX160555 |
| Cossoninae | Onycholipini | Pselactus sp. | CsPse01 | Madeira | EU191839 | EU191871 | H0883552 | H0883780 | H0883868 | KX160755 | KX160706 | KX160628 | KX160643 | KX160556 |
| Cossoninae | Onycholipini | Pseudostenocelis sp. | CsPsc01 | PNG | H0883636 | H0883717 | H0883551 | H0883779 | H0883867 | KU041924 | KU041987 | KU042021 | KU163348 | - |
| Cossoninae | Rhyncolini | Rhyncolus elongatus | CsRhy02 | Norway | - | KU041899 | KU041869 | - | - | - | KU041988 | KU042022 | - | - |
| Cryptorhynchinae | Cryptorhynchinae | Cryptorhynchinae indet sp1 | Crh_sp1 | South Africa | H0883627 | - | - | - | - | KU041920 | KU041982 | - | - | KU041961 |
| Cryptorhynchinae | Cryptorhynchini | Cryptorhynchini indet. sp2 | Crh_sp2 | Cameroon | H0883628 | H0883710 | H0883543 | - | H0883858 | KU041921 | KU041983 | KU042018 | - | KU041962 |
| Curculioninae | Tychiini | Sibinia sp. | CuSib01 | South Africa | H0883649 | H0883725 | H0883563 | - | H0883878 | 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 | C01 | EF-1 $\alpha$ | 28S | CAD | ArgK | PABP1 | UBA5 | lap2 | 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 | H0883652 | H0883728 | H0883567 | - | H0883883 | KU041936 | - | - | - | - |
| Entiminae | Otiorhynchini | Otiorhynchus sulcatus | EnOti02 | Norway | - | KU041904 | KU041875 | KU041887 | - | KU041937 | - | KU042031 | - | - |
| Entiminae | Polydrusini | Polydrusus cervinus | EnPol01 | Norway | H0883653 | H0883729 | H0883568 | H0883793 | H0883884 | - | - | - | - | - |
| Entiminae | Sitonini | Chlorophanus sibiricus | EnChI01 | Russia | H0883651 | H0883727 | H0883566 | - | H0883882 | KU041935 | KU041998 | KU042030 | - | - |
| Entiminae | Sitonini | Sitona lineatus | EnSit01 | Norway | - | KU041905 | KU041876 | KU041888 | KU041883 | - | - | - | - | - |
| Erirhininae | Erirhinini | Himasthlophallus flagellifer | ErHim01 | Russia | H0883654 | H0883730 | H0883569 | - | - | KU041938 | - | - | - | - |
| Lixinae | Lixini | Larinus sp. | CILar01 | Russia | H0883622 | H0883707 | H0883541 | H0883773 | H0883854 | KX160752 | KX160705 | KX160625 | - | - |
| Lixinae | Lixini | Lixus sp. | ClLix01 | Russia | H0883623 | H0883708 | H0883562 | - | H0883855 | - | KU041981 | KU042017 | KU163343 | - |
| Molytinae | Amorphocerini | Amorphocerus rufipes | MoAmo01 | South Africa | H0883664 | H0883736 | H0883575 | H0883803 | H0883893 | KU041940 | KU041999 | KU042033 | - | KU041969 |
| Molytinae | Amorphocerini | Porthetes hispidus | MoPor01 | South Africa | H0883666 | H0883737 | H0883577 | H0883805 | H0883895 | KX160765 | - | KX160634 | KX160650 | - |
| Molytinae | Hylobiini | Hylobius piceus | MoHyl01 | Norway | H0883665 | - | H0883576 | H0883804 | H0883894 | KU041941 | KU042000 | KU042034 | - | - |
| Molytinae | indet. | Molytinae indet.sp. | MoXxx01 | Cameroon | H0883667 | H0883738 | H0883578 | H0883806 | H0883896 | KU041942 | KU042001 | KU042035 | - | KU041970 |
| Molytinae | Psepholacini | Psepholax sp. | Crh_Psx01 | PNG | H0883626 | H0883709 | H0883542 | H0883775 | H0883857 | KU041919 | - | - | KU163345 | - |
| Platypodinae | Platypodini | Platypus impressus | PIPla 07 | Tanzania | - | - | - | - | - | KX160766 | KX160714 | KX160635 | - | KX160564 |
| Platypodinae | Platypodini | Teloplatypus sp1 | PITel01 | CR | - | - | - | - | - | KU041943 | KU042002 | - | KU163354 | - |
| Platypodinae | Platypodini | Teloplatypus sp2 | PITel02 | CR | H0883674 | H0883743 | H0883583 | H0883814 | H0883904 | KU041944 | KU042003 | - | - | - |
| Platypodinae | Platypodini | Triozastus marshalli | PITri02 | Cameroon | KU041895 | - | - | - | - | KX160767 | KX160715 | - | KX160651 | KX160565 |
| Platypodinae | Tesserocerini | Cenocephalus sp. | TsCen01 | CR | H0883682 | H0883751 | H0883593 | H0883826 | H0883912 | KU041945 | KU042004 | - | KU163355 | - |
| Platypodinae | Tesserocerini | Chaetastus tuberculatus | TsCha02 | Cameroon | H0883684 | H0883753 | H0883595 | H0883828 | H0883914 | KX160775 | - | - | KX160654 | - |
| Platypodinae | Tesserocerini | Diapus pusillimus | TsDia01 | PNG | - | - | - | - | - | KU041946 | - | KU042036 | - | KU041971 |
| Platypodinae | Tesserocerini | Diapus unispineus | TsDia02 | PNG | H0883685 | H0883754 | H0883596 | H0883829 | H0883915 | KU041947 | KU042005 | KU042037 | - | KU041972 |
| Platypodinae | Tesserocerini | Genyocerus exilis | TsGen02 | Sarawak | H0883686 | H0883755 | H0883597 | H0883830 | H0883916 | KU041948 | - | KU042038 | KU163356 | - |
| Platypodinae | Tesserocerini | Notoplatypus elongatus | TsNot01 | Australia NSW | H0883688 | H0883757 | H0883599 | H0883832 | H0883918 | - | KU042006 | - | - | - |
| Platypodinae | Tesserocerini | Spathidicerus nobilis | TsSpa01 | PNG | - | KU041906 | KU041877 | - | - | KU041949 | KU042007 | KU042039 | KU163357 | KU041973 |
| Platypodinae | Tesserocerini | Tesserocerus ericerus | TsTes01 | CR | H0883691 | H0883760 | H0883602 | H0883834 | H0883920 | 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 | H0883645 | - | H0883560 | H0883788 | H0883874 | KU041929 | KU041992 | KU042026 | KU163351 | KU041964 |
| Scolytinae | Hylesinini | Dactylipalpus grouvellei | HIDac01 | Ghana | H0883656 | H0883731 | H0883570 | H0883795 | H0883886 | - | - | KU042032 | - | - |
| Scolytinae | Hylesinini | Hylesinus varius | HIHyl02 | Sweden | H0883657 | AF308409 | AF308365 | H0883796 | H0883887 | KX160760 | KX160709 | - | KX160647 | KX160560 |
| Scolytinae | Hylesinini | Phloeoborus sp. | H1Phb02 | Guyana | H0883658 | - | H0883571 | H0883797 | H0883888 | - | - | - | - | KU041968 |
| Scolytinae | Hylurgini | Dendroctonus terebrans | ToDen02 | USA | H0883680 | H0883749 | H0883591 | H0883824 | - | KX160773 | - | - | - | - |
| Scolytinae | Hylurgini | Tomicus piniperda | ToTom01 | Norway | H0883681 | H0883750 | H0883592 | H0883825 | H0883911 | KX160774 | KX160721 | - | KX160657 | KX160569 |
| Scolytinae | Scolytini | Camptocerus aenipennis | ScCam02 | Guyana | H0883676 | H0883745 | H0883587 | H0883818 | H0883907 | KX160769 | KX160717 | KX160637 | KX160652 | - |
| Scolytinae | Scolytini | Cnemonyx vismiaecolens | ScCne01 | Guyana | EU191865 | EU191897 | H0883588 | H0883819 | H0883908 | KX160770 | KX160718 | - | KX160653 | - |
| Scolytinae | Scolytini | Scolytus intricatus | ScScl02 | Sweden | H0883677 | H0883746 | H0883589 | H0883820 | H0883909 | KX160771 | KX160719 | KX160638 | - | - |

of invertebrates, but has been briefly considered for such analyses in vertebrates (Fong \& Fuilta 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-1 $\alpha$ ), the large nuclear ribosomal subunit ( 28 S rDNA), and the mitochondrial gene cytochrome oxidase I (COI).

Nucleotide sequences were blasted in GenBank for verification (minimum E value threshold $=1 \mathrm{E}-4$ ). 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 28 S 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 ( 28 S , 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 $+\mathrm{I}+\Gamma$ ). 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.

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 | lap2 | 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 | $\mathbf{7 2}$ | $\mathbf{4 5}$ | 59 | $\mathbf{3 8}$ | $\mathbf{3 1}$ | $\mathbf{3 1}$ |

likelihood and posterior values) were visualized in the software Tracer 1.6 (Rambaut et al. 2014).

Maximum likelihood analyses were performed by the software IQTREE (Trifinopoulos et al. 2016). Substitution models for each partition were selected using Model Finder (Kalyannamoorthy 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

```
A
Antrib02 AATGTTCCTA GAATCGTACG TGTAGC&--- ------------------------------------ AGCGCCTCCA CCGATCTATA TAAAAACGAA
Antrib04 TCTACGATCA TCССТАGAAC GCCTCCG--- ---------- -------------------------- ACTTCAGGAA GTGAAAGTTA TAGGAACGAA
CdSpr01 AATATTTCGA CTGCACTTCC ATCTTGQAAC AGCAGCAGTA GCAGCAGCAG CACACCATCA TCGTCCGCCG GTGTTGATTA CACGAGCGAG
```



```
MOAmo01 AATATTCCCG CCGTCGTGTT GCCTCCAAGT ATACCATCG- ------------------ TCATCC TCATCGTCCA ACCTAGACTA TAAAGATGAA
TsDia01 AAYGTACCAT CACTAGCCAT ATCCGAQ--- ---------------------------------------------
TsGen02 AATGTTCCAT CACTGCCAGC ATTCGAT--- --------- ----------------------------------- -- CACAAAATTA CAAAAACGAG
Antrib02 RSLNPECPFV VNPSTSGNVP RIVRVA-_-- ------PSAS TDLYKNEEVR LASFENWPAL HIVTPESLAR AGFYYLKEGD NTKCAYCKGV
Antrib04 RTLSPDCPFV VNPSTSISTI IPRTPH----------GTSGS ESYRNEEVRL ASFENWPVTS IVTPESLARA AGFYYLKEGD NTKCAYCKGV
CdSpr01 RALNPQCPFV LNPATSGNIS TALPSQNSS SSSSSTPSSSA GVDYTSEAAR LASFENWPIP HIVSPAALAK SGFYSLKNED NTKCAYCKGV
HlDac01 RLLNPQCPFV LNPATSGNVP SVVTPASVP S--------SS FLXYKNESVR LASFENWPIP HIVAPEDLSR AGFYSLRDGD NTKCAFCKGV
MoAmo01 RTLNPQCPFV LNPATSGNIP AVVLPFSIP S-----SSSSS NLDYKDEAVR LASFESWPIP QIVTPEDLAR SGFYSLRNGD NTKCAFCKGV
TsDia01 RALSPGCRFV LHPSTSGXVP SLAISD--- ---------- IPNYRNEQVR LASFENWPVP HIVTAENLAK AGFYYLKVED KTKCAYCNGV
TsGen02 RALSPRCPFV LCPLTAGNVP SLPAF口-------------- TQNYKNEQVR LASFENWPIP YIVSAEDLAK SGFYYLKVDD KTKCAFCKGV
```

```
B
Antrib02 AATGGTCATC GCCTCCACTC AAATAGTACC GCAAAAGAAC AAGGAT---- -----GACAT CGAGCTTACG CCGGTTCAAG AAAAACTACT
Antrib03 GATGGTCGTG GCCACCTCCC AAGTAGTTCC CCAGAAGAGC AAGGAT---- -----GGCGC CGAGCTCACG GCGGTCCAGG AGAAACTCCT
AtApo02 AATGACTGTG TGCTCAGAAC AAGTGGCACC GCCCAAGAAA GACAAGGACA AGGAGAACCA C СAACTCACA CCGATCCAGG AAAAGCTGTT
BrBre03 GATGGTGGTA GCAAGTATCC AAATAGCCCC CCAGAAGGAA AAGGAT---- --------GC CFAACTAAGT GCAGTCCAGG AAAAGCTTTT
MeMec01 AATGACTCTG GCCACCGTTC AACTGGCACC AGTGAAGAAA TCCGTC--_- -----GTCGA TCATTTCACA CCGGTCCAGG AAAAATTGCT
CdMet01 AATGACCATC GCAACTACCC AGGTAGCACC TACTAGGCGG GAAAAG---- -----GGCGA AGCCCTCACC CCGATCCAGG AGAAGCTCGT
CpCop01 AATGACCATA GCTAGCGCGC AGGTGGCACC CGCCAAACAC TCCAAA---- -----------GG AGAGTTGACG CCGATTCAGG AGAAGCTCGT
Antrib02 GVKFSXEMVI ASTQIVPQKN {D---DIELT PVQEKLLKKM GPAAYPFTFK FPEMAPCSVT LQPGEDDXGK PLGVEYFXKC WVASTEEEKG
Antrib03 GVKFSKEMVV ATSQVVPOKS FD---GAELT AVQEKLLKKM GPQAXPFTFFK FPEMAXCSVT LQPGEDDQGK PLGVEYFVKC WVGSNDEDKG
AtAPO02 GVKFSKEMTV CSEQVAPPKK &KDKENHLLT PIQEKLLKKM GPNAFPFTFR FPDMSPCSVT LQPGEDDQGK PLGVEYYXKC WVGNNEEDKG
BrBre03 GXKFSKEMVV ASIQIAPQKE KD----AELS AVQEKLLKKM GPNAYPFTFH FPDMSPCSVT LQPGEEDQGK PLGVEYYVKC WVGNNEDDKG
MeMec01 GVKFSKEMTL ATVQLAPVKK {V---VDHFT PVQEKLLKKM GPNAYPFTFR FPEMSPCSVT LQPGEDDHGK PLGVEYYVKC WVGNNEEDRG
CdMet01 GVKFSKEMTI ATTQVAPTRR EK---GEALT PIQEKLVKKM GPNAYPFTFN FPDMAPCSVT LQPGEDDQGK PLGVEYFVKC WVGNNEEDKG
CPCop01 XXKFSKEMTI ASAQVAPAKH {K----GELT PIQEKLVKKM GPHAYSFTFH FPDMAPCSVT LQPGEDDQGK PLGVEYYVKC WVGSSDEDKG
```

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 $\mathbf{2}$ - 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 $50-274 \mathrm{bp}$, 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 $50-154 \mathrm{bp}$ 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: 3 $\mathbf{3}^{\text {rd }}$ 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 $50-177 \mathrm{bp}$, 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 $50-795 \mathrm{bp}$ 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 $54-246 \mathrm{bp}$ 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 ( $\mathrm{PP}=1$ ) 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 ( $\mathrm{PP}=1$ ), whereas Mecopelmus grouped with Apion. In the parsimony analysis with third positions excluded (III), Entiminae was recovered as monophyletic $(\mathrm{BS}=55)$, 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 ( $\mathrm{PP}=0.56$ ); these two groups formed the sister lineage to all other Curculionidae ( $\mathrm{PP}=0.51$ ). Among the latter, a monophyletic Entiminae $(\mathrm{PP}=0.62)$ 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 ( $\mathrm{PP}=0.92$ ) 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 $(P P=0.79)$ and was closely related to the molytine tribe Amorphocerini ( $\mathrm{PP}=0.54$ ). 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 ( $\mathrm{PP}=0.95$ ). In the nucleotide analyses of individual genes (Electronic Supplement Figs. S6-S9), the Platypodinae-Dryophthorinae clade was supported by the $\operatorname{ArgK}$ 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; Могімото \& Коліма 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 morpho-logy-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 broadnosed weevils placed among the more advanced Curculionidae. Data on mitochondrial genomes have also shown that Cyclominae and Hyperinae (sensu AlonsoZarazaga \& 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 28 S 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.

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## Electronic Supplement Files

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File 1: mugu\&al-curculionidaephylogeny-asp2018-electronicsup plement-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 28 S , 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-1 $\alpha, \mathrm{CO}, 28 \mathrm{~S}, \mathrm{CAD}$ 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-1 $\alpha$, CAD, 28S and ArgK. - Fig. S9. Tree topologies resulting from the individual Bayesian analyses of amino acid translated COI, EF$1 \alpha$, CAD and ArgK.
File 2: mugu\&al-curculionidaephylogeny-asp2018-electronicsup plement-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.

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Artikel/Article: New molecular markers resolve the phylogenetic position of the enigmatic wood-boring weevils Platypodinae (Coleoptera: Curculionidae) 45-58

