1   The role of the freshwater shrimp *Atyaephyra desmarestii* in leaf litter breakdown in

2   streams

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Abstract

15 16 In aquatic ecosystems, microorganisms and invertebrates provide critical links between 17 plant detritus and higher trophic levels. *Atyaephyra desmarestii* is an omnivorous 18 decapod that inhabits freshwaters and exhibit high tolerance to temperature oscillations 19 and high ability to colonize new habitats. Although A. *desmarestii* is able to ingest a 20 variety of foods, few studies were conducted to elucidate the feeding preferences of this 21 freshwater shrimp on allochthonous detritus. In this study, *A. desmarestii* was allowed 22 to feed on conditioned or non-conditioned alder and eucalyptus leaves in microcosms, 23 in which the animals had access or not to fecal pellets. At the end of the experiment, 24 total body length of the animals was measured, and the remaining leaves and fecal 25 pellets were used for dry mass quantification and assessment of bacterial and fungal 26 diversity by denaturing gradient gel electrophoresis (DGGE). Cluster analyses of DGGE 27 fingerprints indicated that the major differences in microbial communities on leaves or 28 fecal pellets were between leaf types and conditioned and non-conditioned leaves, 29 respectively. The consumption rate by the shrimp did not differ between leaf types, and 30 was significantly higher on leaves conditioned by microorganisms and in treatments 31 without access to feces. In treatments without access to feces, the production of feces 32 and fine particulate organic matter was also significantly higher for conditioned leaves. 33 Overall, our results support the feeding plasticity of *A. desmarestii* and its potential role 34 in plant litter breakdown in streams. This can have implications to maintain stream 35 ecosystem functioning, particularly when typical sensitive shredders decline.

36

37 **Keywords:** *Atyaephyra desmarestii,* consumption rate, detritus quality, fungi, bacteria

38

Introduction

39 40 In freshwater ecosystems, coarse particulate organic matter (CPOM), mainly constituted 41 by fallen leaves and twigs from riparian vegetation, is the major energy input to low- 42 order forested streams (Allan & Castillo, 2007). The decomposition of CPOM is a 43 complex process and extensively conducted by microorganisms and invertebrates 44 (Suberkropp, 1998; Bärlocher, 2005). Both fungi and bacteria play a central role, not 45 only in decomposing plant material, but also transforming it into a more palatable and 46 nutritious food source for invertebrates. Among microorganisms, fungi, especially 47 aquatic hyphomycetes, are reported to dominate microbial decomposition in streams 48 due to notable adaptations such as the ability to i) produce large amounts of sigmoid and

tetraradiate conidia that facilitates adhesion to substrates in turbulent waters (Bärlocher, 2009), ii) be active at low temperatures, and iii) produce lignocellulose-degrading enzymes that break the structural compounds of plant material (Suberkropp, 1998; Bärlocher, 2005).

Although in streams there are several functional feeding groups of benthic invertebrates (Cummins & Klug, 1979), a major role in litter breakdown is attributed to shredders. Shredders have mouthparts adapted for the maceration of CPOM converting it to fine particulate organic matter (FPOM), which can be consumed by collectors ensuring the transference of carbon and energy in aquatic detritus food webs (Graça, 2001; Alan & Castillo, 2007). The feeding behaviour of many shredder species on plant litter is well established (Graça et al., 2001). However, several typical shredders are sensitive to environmental change, such as eutrophication (Pascoal et al., 2003; 2005), temperature increase (Ferreira et al., 2010) and alteration in riparian vegetation (Graça et al., 2002). Plant litter decomposition may be compromised when shredders decline, unless more tolerant species with broader feeding plasticity will drive the process. In eutrophic temperate streams, the decline of shredders on decomposing leaves can be accompanied by an increased density of oligochaetes which contribute to litter breakdown due to their movements and feeding behaviour (Pascoal et al. 2003; 2005). Also, in tropical streams, where typical shredders are scarce or absent, organic matter breakdown is driven by decapods able to play a range of trophic roles (e.g. Crow et al., 2001; Covich et al., 2003).

Atyaephyra desmarestii Millet (1831) is a small decapod with a wide geographic distribution in freshwater habitats, spanning from North Africa to the Middle East, a large part of Europe and some Mediterranean islands (Muñoz et al., 2009; Straka & Špaček, 2009). In Portugal, this species occurs in different habitats, such as rivers, temporary streams, reservoirs, rice fields, lakes, and coastal lagoons (Fidalgo & Gerhardt, 2003). A variety of food sources, namely algae, mud, fecal pellets and plant litter, are reported to be used by *A. desmarestii* (Fidalgo, 1985; 1990a; 1990b; Fidalgo & Gerhardt, 2003; Callisto, 2006), and this species constitutes an important food item for fish (García-Berthou & Moreno-Amich, 2000). Because this species is quite tolerant to temperature and salinity variations and is very successful in colonizing new aquatic environments (Gauthier, 1924; Van den Brink & Van der Velde, 1986; Fidalgo, 1989; Fidalgo & Gerhardt, 2003; Janssens de Bisthoven et al., 2006; Straka & Špaček, 2009), we expect that *A. desmarestii* may occupy vacant ecological niches when sensitive

83   invertebrates, such as shredders, decline. This may be particularly relevant under the

84   ongoing global change. However, few studies were conducted to assess the role of this

85   freshwater shrimp in organic matter turnover (but see Calisto 2006). To elucidate the

86   feeding preferences of *A. desmarestii* for particulate organic matter, conditioned or non-

87   conditioned leaves of alder and eucalyptus were exposed to the shrimps in microcosms,

88   in which the animals had access or not to feces. After 16 days, we determined leaf

89   consumption and fecal production by *A. desmarestii*, and fungal and bacterial diversity

90   associated with leaf litter and fecal pellets.

91

92   **Methods**

93   Microcosm setup

94   The shrimps were collected with a hand net (2 mm mesh size) near submerged

95   macrophytes (*Ceratophyllum* sp., *Elodea* sp., *Myriophyllum* sp. and *Potamogeton* sp.) at

96   the left bank of the Minho River (northern Portugal) at about 12 km upstream the mouth

97   of the river. The animals were acclimated to the laboratory for two weeks prior the

98   experiment (water temperature: 15 ± 1 ºC; photoperiod: 12 h light and 12 h dark). The

99   acclimation was done in glass aquaria containing stream water diluted 1:2 with

100   dechlorinated tap water, under constant aeration. The stream water had pH = $7.64 \pm$

101   0.23, conductivity = 84.00 ± 1.41 µS cm^{-1}, total dissolved solids = 40.50 ± 3.54 mg L^{-1},

102   hardness = 31.00 ± 1.41 mg CaCO$_3$ L^{-1}, nitrate = 0.57 ± 0.06 mg N-NO$_3$ L^{-1},

103   orthophosphate = 0.12 ± 0.01 mg P-PO$_4$ L^{-1}, planktonic chlorophyll *a* = 0.40 ± 0.19 mg

104   m^{-3}. During acclimation, animals were fed *ad libitum* on mud, aquatic macrophytes,

105   tetramin and leaves of *Alnus glutinosa* (L.) Gaertn (alder) and *Eucalyptus globulus*

106   Labill (eucalyptus). The animals were starved for two days before the beginning of the

107   experiment.

108   Leaves of alder and eucalyptus, collected in October 2008, were leached for one day

109   and cut into 12 mm diameter disks. Sets of leaf disks were placed in fine-mesh bags (0.5

110   mm; 20 x 20 cm) and immersed in a stream on 25th November 2008 to allow microbial

111   colonization. After 7 days of immersion, the bags were brought to the laboratory and

112   colonized leaf disks were washed to remove debris prior to be placed in microcosms.

113   Microcosms consisted of plastic vessels with 200 mL of filtered (140 µm-pore size

114   membrane) stream water diluted 1:2 as indicated above. Half of the microcosms

115   contained a plastic mesh (1 mm pore size) to separate shrimps and leaf disks from the

116   bottom of the vessel and prevent coprophagy. One animal and ten leaf disks were placed

117   into each microcosm according to the following treatments (15 replicates): leaf type

118   (alder *versus* eucalyptus), microbial conditioning (colonized *versus* non-colonized

119   leaves) and coprophagy (animal with access or not to feces). Microcosms without

120   shrimps were used to correct leaf mass loss due to factors other than feeding.

121   Microcosms were kept under constant aeration at 15 ± 1 ℃ with a photoperiod of 12 h

122   light and 12 h dark for 16 days. Half of the water in each microcosm was replaced every

123   two days. At the end of the experiment, the total animal length was measured; leaf

124   disks, feces and other FPOM were collected and freeze dried for dry mass quantification

125   and microbial diversity assessment.

126

127   Animal biomass

128   Animal dry mass was estimated based on the animal length, measured from the tip of

129   the rostrum to the end of the uropods, as follows: $\ln W = -0.49 + 0.14\ L$, where W is the

130   animal dry weight in mg and L is the total body length of the animal in mm (Fidalgo,

131   1983).

132

133   Leaf consumption and fine particulate organic matter production

134   Leaf disks, and fecal pellets and other FPOM were freeze-dried for two days and

135   weighed to the nearest 0.01 mg. Consumption rates were calculated as the difference

136   between dry mass of leaves unexposed and exposed to the animal divided by the

137   exposure time (16 days). Values were expressed as mg leaf dry mass per animal per

138   day. Fecal and FPOM production rates were determined as mg of feces and FPOM,

139   respectively, per animal per day.

140

141   Microbial diversity

142   DNA was extracted from four freeze-dried leaf disks (randomly selected from eight

143   replicates) and from ca. 25 mg of fecal pellets with a soil DNA extraction kit (MoBio

144   Laboratories, Solana Beach, California), according to the manufacturer instructions. The

145   ITS2 region of fungal ribosomal DNA was amplified with the primer pair ITS3GC and

146   ITS4 (Duarte et al., 2010), while bacterial ribosomal DNA was amplified with the

147   primer pair 338GC and 518 (Duarte et al., 2010). For PCR of fungal and bacterial DNA,

148   2x of GoTaq® Green Master Mix (Promega), 0.4 µM of the appropriate primers and

149   1 µL of DNA were used in a final volume of 25 µL.

PCRs were carried out in a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) using the following program: initial denaturation at 95 °C for 2 min; 36 cycles of denaturation at 95 °C for 30 s; primer annealing at 55 °C for 30 s and extension at 72 °C for 1 min; and final extension at 72 °C for 5 min (Duarte et al., 2010). Denaturing gradient gel electrophoresis (DGGE) was performed using a DCode^{TM} Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA). For fungi, 700 ng of the amplified DNA products with 380-400 bp were loaded on 8% (w/v) polyacrylamide gel in 1x Tris-acetate-EDTA (TAE) with a denaturing gradient from 30 to 70% (100% denaturant corresponds to 40% formamide and 7 M urea). For bacteria, 700 ng of the amplified DNA products with 200 bp were loaded on 8% (w/v) polyacrylamide gel in 1x TAE with a denaturing gradient from 40 to 75%. The gels were run at 55 V, 56 °C for 16 h and stained with 1x of GelStar (Lonza, Rockland, Inc. USA) for 10 min. The gel images were captured under UV light in a gel documentation system GenoSmart (VWR International, East Grinstead, UK).

Statistical analyses

A three-way ANOVA was used to test the effects of leaf type (alder or eucalyptus), microbial conditioning (colonized or non-colonized leaves) and coprophagy (animal with access or not to feces) on animals dry mass and leaf consumption rates. A two-way ANOVA was used to test the effects of leaf type and microbial conditioning on fecal and other FPOM production by the shrimps.

The DGGE gel was aligned and normalized with GelCompar II (Applied Maths, Belgium), and cluster analyses of banding patterns were done using the unweighted pair group method average (UPGMA) based on the Dice coefficient of similarity.

Results

Leaf consumption rate by *A. desmarestii* varied between 0.01 and 0.75 mg leaf dry mass animal^{-1} d^{-1} for non-conditioned alder leaves with access to feces and for conditioned eucalyptus leaves without access to feces, respectively (Fig. 1). Both microbial conditioning and access to feces affected leaf consumption rates by the animals (3-way ANOVA, P<0.0001 and P=0.0002, respectively; Table 1). The highest consumption rates were found in microcosms with conditioned leaves in which the shrimp had no access to feces, no matter the leaf type.

183   Fecal production rates varied between 0.24 and 0.44 mg dry mass animal^{-1} d^{-1} for non-

184   conditioned eucalyptus leaves and conditioned alder leaves, respectively, when shrimps

185   had no access to feces (Fig. 2A). Fecal production rates were significantly affected by

186   microbial conditioning but not by leaf type (2-way ANOVA, P=0.01 and P=0.3,

187   respectively; Table 2). The production of fine particulate organic matter (FPOM),

188   excluding the feces, varied between 0.04 and 0.12 mg animal^{-1} d^{-1} for non-conditioned

189   eucalyptus leaves and conditioned alder leaves in microcosms without access to feces

190   (Fig. 2B). FPOM production rates were significantly affected by both leaf type and

191   microbial conditioning (2-way ANOVA, P=0.0003 and P=0.04, respectively; Table 2).

192   At the end of the experiment, mean shrimp biomass varied between 8.9 mg for non-

193   conditioned alder leaves with access to feces and 11.7 mg for non-conditioned

194   eucalyptus leaves with access to feces (Fig. 3), but these differences were not significant

195   (3-way ANOVA, P>0.05; Table 1). However, the interaction between leaf type and

196   access to feces was significant (P=0.02).

197   The analysis of fungal communities based on PCR-DGGE showed a higher number of

198   operational taxonomic units (OTUs) on leaves (up to 33 OTUs on conditioned alder

199   with access to feces) than in feces (up to 15 OTUs in microcosms with conditioned

200   alder leaves) (Fig. 4A). Cluster analysis of fungal DGGE fingerprints showed the

201   presence of two major groups: fungal communities on leaves and feces in eucalyptus

202   microcosms separated from those in alder microcosms (Fig. 4B). In each group, and

203   especially for alder, communities on leaves and feces in microcosms with conditioned

204   leaves separated from those with non-conditioned leaves (Fig. 4B).

205   A high number of bacterial OTUs was associated with both leaves and feces (up to 38

206   OTUs on conditioned alder leaves with access to feces) (Fig. 5A). Cluster analysis of

207   bacterial DGGE fingerprints indicated the presence of two major groups: communities

208   on leaves and feces from microcosms containing conditioned eucalyptus leaves

209   separated from the remaining treatments. Further, bacterial communities on leaves or

210   feces in microcosms with conditioned alder leaves separated from those with non-

211   conditioned leaves (Fig. 5B).

212

213   **Discussion**

214   In the present study, the shrimp *A. desmarestii* was able to feed on alder or eucalyptus

215   leaves and the leaf type did not affect its consumption rate. Moreover, consumption

216   rates of conditioned leaves were 75 times higher than those of non-conditioned leaves.

Microorganisms when growing on leaves degrade the recalcitrant polymers of plant-cell wall and immobilize nutrients (e.g., nitrogen or phosphorus) either from plant material or surrounding water, making plant litter more nutritious to consumers (Suberkropp, 1998). Several species of shredders are likely to selectively ingest fungal patches on leaves (Bärlocher, 1985; Arsuffi & Suberkropp, 1988); consequently, leaf conditioning can be even more important than leaf type in determining food preference and consumption rate by invertebrates (Graça et al., 2001).

The lack of preference between leaf types was somehow surprising because lower consumption rates of eucalyptus relatively to alder are reported for typical shredder species. Indeed, in the laboratory, the shredders *Sericostoma vitattum* and *Tipula lateralis* rejected conditioned eucalyptus leaves over other leaf species (Canhoto & Graça, 1995; 1999). Eucalyptus leaves contain large amounts of oils, polyphenols and a waxy cuticle that can restrain leaf consumption by invertebrate shredders (Canhoto & Graça, 1999). However, Chironomidae larvae caused considerable mass loss of eucalyptus leaves, which was attributed to their small size that allow these animals to selectively consume leaf mesophyll while leaving the oil glands intact (Canhoto & Graça, 1999). The putative ability of *A. desmarestii* to avoid oil glands when feeding on eucalyptus leaves is currently unknown and is worth of further investigation.

In our study, consumption rates of conditioned alder leaves (0.67 mg leaf dry mass animal^{-1} d^{-1}) by the shrimp *A. desmarestii* were lower than previously reported (1.24 mg leaf dry mass animal^{-1} d^{-1}; Callisto, 2006). This may be due to differences in i) time of leaf immersion in the stream (1 week in our study *versus* 3 weeks in the cited study) and/or ii) water chemistry used in the experiment, aspects that are known to affect microbial colonization of leaf litter (Pascoal et al., 2005). Indeed, the amount of microbial biomass and the presence of certain microbial taxa on leaves are considered important factors for determining food preferences of invertebrate shredders (Suberkropp, 1998; Graça, 2001; Alan & Castillo, 2007).

Here, the production of feces by *A. desmarestii* varied between 0.24 and 0.44 mg dry mass animal^{-1} d^{-1} in non-conditioned eucalyptus and conditioned alder leaves, respectively. These values are similar to those found in a previous study in which *A. desmarestii* fed on conditioned alder leaves (0.25 mg dry mass animal^{-1} d^{-1}; Callisto, 2006). Many consumers of CPOM produce large amounts of feces that are the dietary mainstay for some animals, while for others, feces work as a food supplement (Frankenberg & Smith, 1967; Wotton, 1980). Coprophagy has been reported in *A.*

251 *desmarestii* (Fidalgo, 1990b; Fidalgo & Gerhardt, 2003) and this was supported in our
252 study by an increased consumption of both leaf types when animals had no access to
253 feces. The production of fecal pellets and other FPOM was higher in treatments with
254 conditioned than non-conditioned leaves. Besides the production of FPOM by shrimp
255 feeding activities, microorganisms can also directly contribute to the fragmentation of
256 leaf material and the production of FPOM (Suberkropp, 1998). Additionally, we found
257 higher FPOM production from alder than eucalyptus leaves, which may be explained by
258 the high recalcitrance of eucalyptus leaves (Abelho & Graça, 1996).

259 In our study, the differences in leaf consumption and production of feces and other
260 FPOM by *A. desmarestii* were somehow supported by the structure of microbial
261 communities on leaves, as indicated by cluster analyses of DNA fingerprints of fungi
262 and bacteria. Although substrate is sometimes reported as a minor factor structuring
263 fungal communities (Nikolcheva & Bärlocher, 2005; Das et al., 2007), we found that
264 fungal communities on leaves or in feces in alder microcosms clearly differed from
265 those in eucalyptus microcosms. This separation may mirror differences in leaf quality,
266 since eucalyptus leaves have physical barriers and compounds that retard the
267 colonization and growth of some aquatic hyphomycete species (Canhoto & Graça,
268 1999).

269 We also found that communities of fungi or bacteria on leaves that were previously
270 conditioned in the stream differed from those on non-conditioned leaves. This was
271 expected because recently fallen leaves carry terrestrial fungi (e.g. Nikolcheva et al.,
272 2005) and bacteria that may remain on non-conditioned leaves. But when leaves are
273 immersed in a stream, aquatic species may take advantage leading to shifts in species
274 composition. The number of fungal OTUs was lower in non-conditioned leaves than in
275 colonized leaves, but such differences were not found for bacteria. The high number of
276 bacterial OTUs on non-conditioned leaves can be partially explained by the differences
277 between fungi and bacteria. Bacteria are typically suspended in or attached to a
278 substrate, and its presence on decomposing leaves do not necessarily mean that they are
279 having a role in litter decomposition; substrates can also be used for settling down. On
280 the contrary, saprotrophic fungi are typically filamentous and actively participate in
281 decomposition by the penetration of their hyphae in the substrates (Suberkropp, 1998).

282 In our study, microbial diversity on feces was high (as number of DGGE OTUs), and
283 the structure of fungal and bacterial communities was similar in fecal pellets and leaves.
284 In a recent study, the extraction, amplification and sequencing of DNA from feces of

285 two detritus feeding amphipods also revealed the presence of numerous fungal
286 phylotypes (Sridhar et al., 2010). The authors were able to recover up to 5 aquatic
287 hyphomycete species from invertebrate feces out of 10 species that were colonizing the
288 leaves (Sridhar et al., 2010). Also in our study, the number of fungal DGGE OTUs was
289 lower on feces than on leaves. However, we did not determine which DNA sequences
290 were assigned to aquatic microbes, therefore we cannot discard the hypothesis that some
291 fungal or bacterial OTUs on feces might belong to gut symbionts (Sridhar et al., 2010).
292 Overall results show that the freshwater shrimp *A. desmarestii* was able to consume
293 both alder and eucalyptus leaves at similar rates. Conditioned leaves were preferred
294 over non-conditioned leaves, and feces appeared to be a nutritious food source for this
295 freshwater shrimp. Hence, our results support the feeding plasticity of *A. desmarestii*.
296 Although this shrimp is generally associated with high quality waters, it has been found
297 at sites with compromised water quality (Oscoz & Durán, 2005). Moreover, this shrimp
298 is reported to tolerate notable temperature oscillations (Van den Brink & Van der Velde,
299 1986) and to have a high ability to colonize new freshwater habitats (Van den Brink &
300 Van der Velde, 1986; Oscoz & Durán, 2005; Straka & Špaček, 2009). Because typical
301 shredders are generally sensitive to water quality and warming temperature, their
302 populations may decline under the ongoing global change with impacts on plant litter
303 decomposition in streams (Graça et al., 2002; Pascoal et al., 2003; 2005; Ferreira et al.,
304 2010). For these reasons, it is conceivable that *A. desmarestii* may play a greater role in
305 plant litter breakdown in streams in the near future. Indeed, in some insular streams,
306 freshwater shrimps are the main drivers of litter breakdown following the riparian
307 inputs, acting as shredders, and are well adapted to disturbances rapidly re-colonizing
308 headwater streams following droughts and floods (Crow et al., 2001; Covich et al.,
309 2003).
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428   **Tables**

429   **Table 1.** Effects of leaf type, microbial conditioning and access to feces on the biomass

430   of *A. desmarestii* and leaf consumption rates. Values were compared by a 3-way

431   ANOVA.

Treatment	Animal biomass			Leaf consumption rates		
	df	F	P	df	F	P
Leaf type	1	0.2	0.6	1	2.4	0.1
Microbial conditioning	1	1.5	0.2	1	30.9	<0.0001
Access to feces	1	1.1	0.3	1	15.3	0.0002
Leaf type* microbial conditioning	1	0.9	0.3	1	0.03	0.8
Leaf type*access to feces	1	5.7	0.02	1	0.1	0.7
Microbial conditioning*access to feces	1	0.04	0.8	1	0.5	0.5
Leaf type*microbial conditioning*access to feces	1	0.2	0.7	1	0.05	0.8

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Table 2. Effects of leaf type and microbial conditioning on fecal and other fine particulate organic matter (FPOM) production by *A. desmarestii*. Values were compared by a 2-way ANOVA.

Treatment	Fecal production			FPOM production		
	df	F	P	df	F	P
Leaf type	1	1.0	0.3	1	14.6	0.0003
Microbial conditioning	1	6.4	0.01	1	4.4	0.04
Leaf type*microbial conditioning	1	0.09	0.8	1	0.2	0.6

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Figure legends

Figure 1. Leaf consumption rates by *A. desmarestii* in microcosms with conditioned and non-conditioned leaves of alder (white bars) and eucalyptus (black bars), with access or not to feces. Mean ± SEM, n =15.

Figure 2. Production rates of feces (A) and other fine particulate organic matter (FPOM) excluding feces (B) in microcosms with *A. desmarestii* feeding on conditioned or non-conditioned leaves of alder (white bars) and eucalyptus (black bars). Mean ± SEM, n =15.

Figure 3. Biomass of *A. desmarestii* after 16 days in microcosms with conditioned and non-conditioned leaves of alder (white bars) and eucalyptus (black bars), with access or not to feces. Mean ± SEM, n =15.

Figure 4. DGGE fingerprints (A) and cluster dendograms (B) of fungal communities on leaves (L) and *A. desmarestii* feces (F). Dendograms were constructed from UPGMA analysis based on the Dice coefficient of similarity. M, mixture of DNA of 5 aquatic hyphomycete pure cultures.

Figure 5. DGGE fingerprints (A) and cluster dendograms (B) of bacterial communities on leaves (L) and *A. desmarestii* feces (F). Dendograms were constructed from UPGMA analysis based on the Dice coefficient of similarity. M, mixture of DNA of 5 bacterial pure cultures.