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# Coral Genus Differentiation Based on Direct Analysis in Real Time-High Resolution Mass Spectrometry-Derived Chemical Fingerprints

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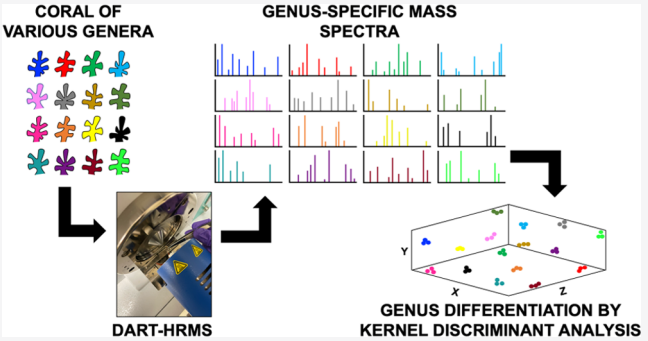
**ABSTRACT:** Coral reefs are one of the most biologically diverse ecosystems, and the accurate identification of the species is essential for diversity assessment and conservation. Current genus determination approaches are time-consuming and resource-intensive and can be highly subjective. To explore the hypothesis that the small-molecule profiles of coral are genus-specific and can be used as a rapid tool to catalogue and distinguish between coral genera, the small-molecule chemical fingerprints of the species *Acanthastrea echinata, Cataphyllia jardinei, Duncanopsammia axifuga, Echinopora lamellosa, Euphyllia divisa, Euphyllia paraancora, Euphyllia paradivisa, Galaxea fascicularis, Herpolitha limax, Montipora confusa, Monitpora digitata, Montipora setosa, Pachyseris rugosa, Pavona cactus, Plerogyra sinuosa, Pocillopora acuta, Seriatopora hystrix, Sinularia dura, Turbinaria peltata, Turbinaria reniformis, Xenia elongata,* and *Xenia umbellata* were generated using direct analysis in real time-high resolution mass spectrometry (DART-HRMS). It is demonstrated here that the mass spectrum-derived small-molecule profiles for coral of different genera are distinct. Multivariate statistical analysis processing of the DART-HRMS data enabled rapid genus-level differentiation based on the chemical composition of the coral. Coral samples were analyzed with no sample preparation required, making the approach rapid and efficient. The resulting spectra were subjected to kernel discriminant analysis (KDA), which furnished accurate genus differentiation of the coral. Leave-one-out cross-validation (LOOCV) was carried out to determine the classification accuracy of each model and confirm that this approach can be used for coral genus attribution with prediction accuracies ranging from 86.67 to 97.33%. The advantages and application of the statistical analysis to DART-HRMS-derived coral chemical signatures for genus-level differentiation are discussed.




## INTRODUCTION

Notwithstanding the fact that they occupy less than 1% of the planet's surface, coral reefs exhibit greater species diversity than any other ecosystems on earth,[1] and it is estimated that upward of 25% of identified marine species are associated with coral reefs.[2] By virtue of serving as biodiversity habitats for multiple species; bellwethers of climate change, environmental pollution, and the health of aquatic ecosystems; and reservoirs of novel biologically active natural products, they are of high environmental and economic importance.[3] Consequently, the rapid and precipitous decline in the coral population over the past few decades due to their high susceptibility to damage caused by climate change, pollution, overfishing,[2,4,5] and predation by the crown-of-thorn starfish *Acanthaster plancii* have raised alarm.

The rapid disappearance of reef ecosystems confers an urgency to the need to define and catalogue coral diversity because the development and implementation of effective conservation policies depend on accurate biodiversity assessment[6] and hinge on a sound understanding of the influence and impact of biological species diversity, biogeography, animal physiology, community ecology, and evolution[7,8] in coral reefs. However, in many instances, the precise assessment of the conservation status of corals is rendered difficult by the lack of a reliable taxonomic framework. This challenge is a consequence of certain attributes inherent to many coral species that make the task of species delimitation extremely challenging. To date, many reported species assignments are plagued with inaccuracies and uncertainty.[9,10] Historically, coral systematics and taxonomy have been based almost exclusively on morphological features of the skeleton (i.e., the corallum).[11−13] These include colony form (laminar, encrusting, massive, and branching), arrangement and size of protrusions between corallites, the characteristics of the septa and cortae,[12] and the shape and dimensions of sclerites.[9,10,14] This morphologically based assessment also requires the

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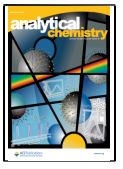






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sacrifice of some coral to accomplish microscopic examination of the coral sclerites. While morphology-based identification remains invaluable for taxonomic description and field identification, morphology-based approaches often fail to detect speciation in the absence of morphological variation[7,15] and do not account for the observation that corals can modify their skeletal morphology to cope with variation among habitats and environmental shifts over time[16−20] (i.e., exist as ecomorphs). This morphological plasticity can be extensive, often generating skeletal features that overlap between species.[13,16,17] Thus, the macromorphological characters that underpin traditional taxonomy are notoriously variable between reef habitats and biogeographic regions, leading to phenotypic overlap between distantly related taxa (i.e., homoplasy) that confounds phylogenetic analyses.[13,17] The result is that skeletal features that are widely used in identifying species of corals are not fully reflective of evolutionary relationships within families and even between conspecific populations.[12,19,21,22] This has led to considerable confusion in the taxonomy and systematics of scleractinian corals[16,17,19,23] (i.e., stony or hard corals in the phylum *Cnidaria* that build hard skeletons) and an overestimation of the number of species contained within various genera, including the *Pocillopora*.[13,24]

In contrast to species delimitation based on macromorphological features, the technique of DNA barcoding is capable of resolving complex phylogenetic relationships that include hidden diversity, introgression, reticulate evolution, or hybridization.[7,25−27] DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species.[28] A 658-bp region of the mitochondrial (mt) cytochrome *c* oxidase subunit I (COI) gene has been widely used in diverse applications of DNA barcoding in animals.[29] Phylogenetic analyses of COI across a wide range of scleractinian species shows that it is consistent with coral taxonomy to the genus level[19,22] and even to the species level in multiple genera including *Stylophora*,[30] *Acropora*,[31] *Orbicella*,[32] *Montipora*,[33] *Platygyra*,[34] *Pocillopora*,[16,24,35,36] *Seriatopora*,[37] *Porites*,[20] and *Stylophora*,[30] among others, suggesting that COI can serve as a genetic tool for broadening the taxonomic resolution of corals. Recent studies that have integrated molecular and micromorphological/structural evidence have found correspondence between molecular-based and morphologically based taxa.[38] This approach has also resolved outstanding problems in coral taxonomy from the family-level[21] to the species level.[39] For example, in the last decade, a growing body of literature has focused on resolving the taxonomy of *Pocillopora* by assessing morphological traits in conjunction with genetic markers.[16,35,36,40] These studies have identified the mitochondrial open reading frame (mtORF) as an efficient marker for delineating the *Pocillopora* species. The mtORF marker has been recently used by Johnston et al.[41] in conjunction with a genus-wide genomic comparison of *Pocillopora*, which confirmed it as a suitable and fast tool for delineating most *Pocillopora* species. Nevertheless, the application of molecular systematics (i.e., DNA barcoding) approaches to the study of coral species boundaries have been only partially successful.[9,10] While these techniques have revealed that some well-known morphospecies comprise cryptic species complexes,[42] it is also the case that numerous morphologically distinct species share identical haplotypes at barcoding loci.[10,43] For example, because mitochondrial genes evolve slowly in *Anthozoa*,[44] these markers often simply lack the resolution to distinguish recently diverged species.[45] As a result, it is often not possible to conclude with certainty whether morphologically distinct individuals that share identical DNA barcodes represent different octocoral species or morphological variants of a single species.[9] In addition, the reported ability of some species of *Sinularia* to hybridize in the laboratory[9] raises the possibility that naturally occurring hybridization events could contribute to the observed morphological diversity of this genus, as has been suggested for stony corals.[46] Correlating genetic signatures to morphology in *Pocillopora* has proven difficult due to incongruencies between whole colony morphology and genetic lineages.[47] Thus, some doubts have been expressed about the sole use of either of these techniques.[13,47] These challenges are compounded by the time- and resource-intensiveness of the experimental process associated with the development of the barcodes.

Corals are well-known repositories of unique bioactive compounds. For example, many *Sinularia* species produce secondary metabolites used for allelopathy and predator deterrence,[9,48] making the genus a rich and diverse source of bioactive natural products.[49] These molecules serve as biomarkers for the respective species in which they are found, and their discovery hints at the possibility that there may be genus- and species-specific chemical profiles that have the potential to serve as a tool that can be used for genus and species identification and differentiation.[50−52] Such metabolome profiles have been shown to be correlated to species identity and/or can be used for genus and even species-level classification of endangered wildlife, including pangolins[53] and Brazilian redwoods,[54] necrophagous insects,[55] and psychoactive plants.[56] This approach involves the generation of metabolome profiles by a spectroscopic or mass spectrometric technique, the outputs of which are processed by multivariate statistical analysis to generate prediction models against which unknown samples can be screened to glean genus- or species-level information.[57] One of the most efficient approaches to accomplish this is by chemometric processing of the data generated from sample analysis by direct analysis in real time-high resolution mass spectrometry (DART-HRMS). Using this technique, samples can be analyzed in their native form, often without the requirement for pretreatment or processing. Because the analysis is commonly performed under soft ionization conditions, the output of a typical analysis is a mass spectrum with peaks representative of the unfragmented protonated precursors of all of the molecules that are detected. The range of compounds spans the dielectric constant spectrum and, for the analysis of complex unprocessed matrices in particular, has been shown to yield species-specific chemical profiles that can serve as the basis for prediction of species identity.[53−57] Importantly, each analysis takes ∼5 s, and thus, the hundreds or thousands of sample replicates required to create species-specific databases against which unknown samples can be screened to learn of their species identity can be rapidly created. Thus, while it would be ideal to be able to utilize DNA barcoding approaches for genus identification, alternative methods that can accomplish this task more rapidly and in the short term would be of value.

Herein, we describe the DART-HRMS analysis of 22 coral species representing 16 genera. Chemometric processing of the generated mass spectra revealed that the genera exhibited genus-specific chemical fingerprints that have the potential to be used for differentiation.


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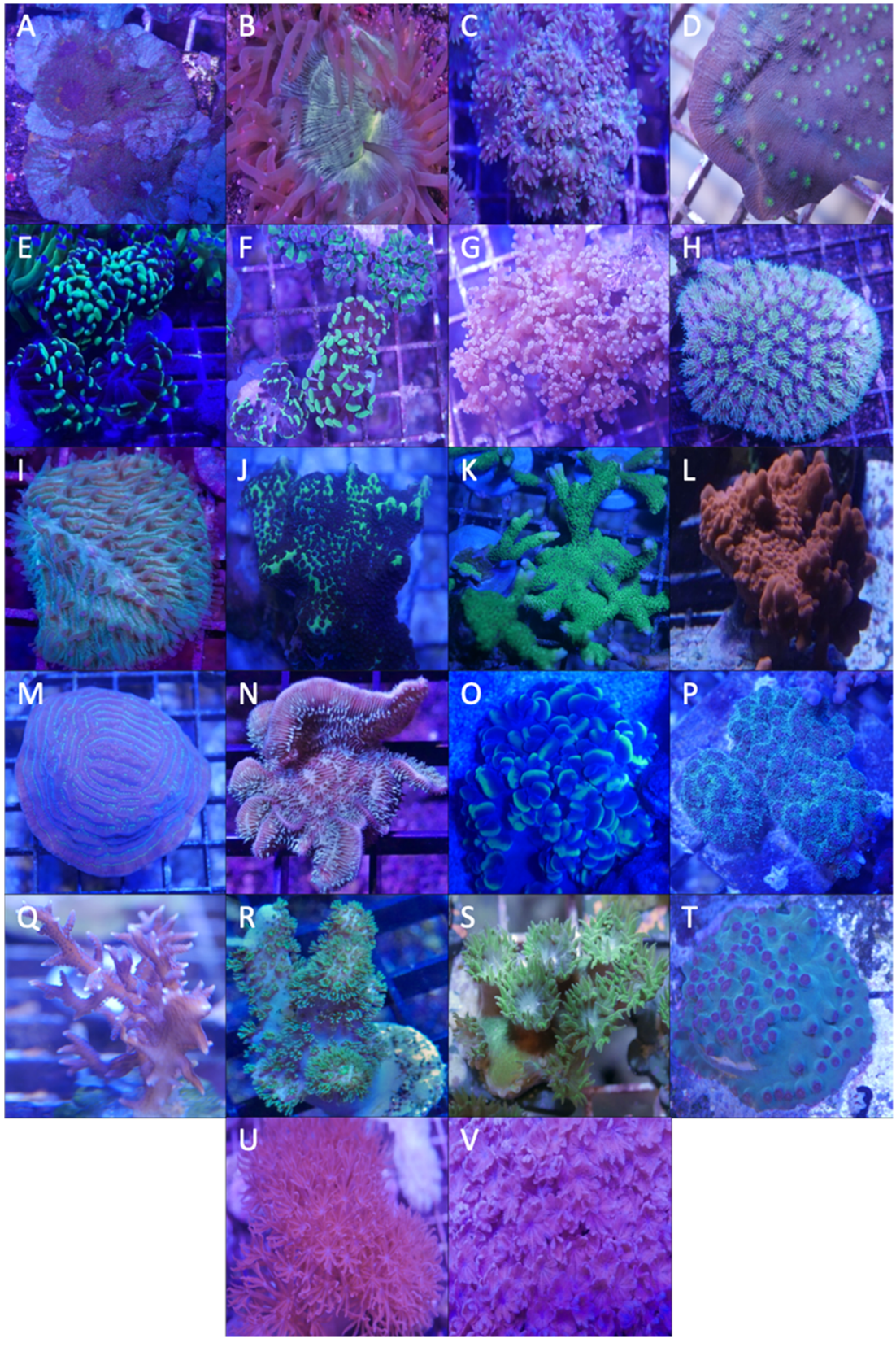


**Figure 1.** Images of coral species included in this study. (Panel A): *A. echinata*; (Panel B): *C. jardinei*; (Panel C): *D. axifuga*; (Panel D): *E. lamellosa*; (Panel E): *E. divisa*; (Panel F): *E. paraancora*; (Panel G): *E. paradivisa*; (Panel H): *G. fascicularis*; (Panel I): *H. limax*; (Panel J): *M. confusa*; (Panel K): *M. digitata*; (Panel L): *M. setosa*; (Panel M): *P. rugosa*; (Panel N): *P. cactus*; (Panel O): *P. sinuosa*; (Panel P): *P. acuta*; (Panel Q): *S. hystrix*; (Panel R): *S. dura*; (Panel S): *T. peltata*; (Panel T): *T. reniformis*; (Panel U): *X. elongata*; and (Panel V): *X. umbellata*.



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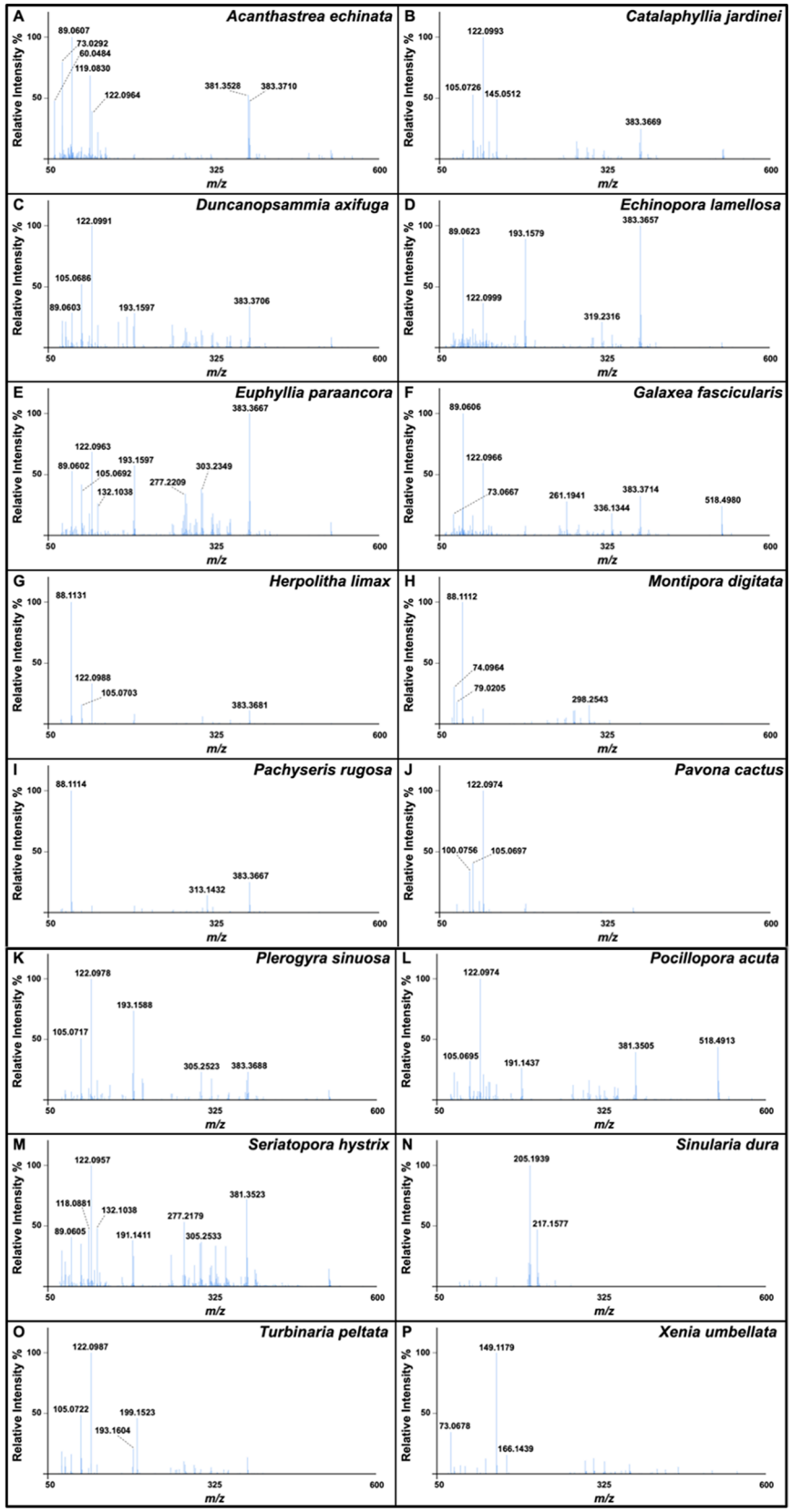


**Figure 2.** Soft ionization DART mass spectra acquired in the positive-ion mode for the 16 studied genera. (Panel A): *Acanthastrea*; (Panel B): *Catalaphyllia*; (Panel C): *Duncanopsammia*; (Panel D): *Echinopora*; (Panel E): *Euphyllia*; (Panel F): *Galaxea*; (Panel G): *Herpolitha*; (Panel H): *Montipora*; (Panel I): *Pachyseris*; (Panel J): *Pavona*; (Panel K): *Plerogyra*; (Panel L): *Pocillopora*; (Panel M): *Seriatopora*; (Panel N): *Sinularia*; (Panel O): *Turbinaria*; and (Panel P): *Xenia*. The mass measurement data corresponding to each spectrum are presented in SI Tables S-1−S-16.


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Table 1. Prominent $m/z$ Values Observed in the Representative Spectra of Each Genus[a]

| Genus of Representative Spectrum | Prominent $m/z$ Values | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 60 | 73 | 74 | 79 | 88 | 89 | 100 | 105 | 118 | 119 | 122 | 132 | 145 | 149 | 166 | 191 | 193 | 199 | 205 | 217 | 261 | 277 | 298 | 303 | 305 | 313 | 319 | 336 | 381 | 383 | 518 |
| *Acanthastrea* | ✓ | ✓ | | | | ✓ | | | | ✓ | ✓ | | | | | | | | | | | | | | | | | | ✓ | ✓ | |
| *Catalaphyllia* | | | | | | | | ✓ | | | ✓ | | ✓ | | | | | | | | | | | | | | | | | ✓ | |
| *Duncanopsammia* | | | | | | ✓ | | ✓ | | | ✓ | | | | | | ✓ | | | | | | | | | | | | | ✓ | |
| *Echinopora* | | | | | | ✓ | | | | | ✓ | | | | | | ✓ | | | | | | | | | | ✓ | | | ✓ | |
| *Euphyllia* | | | | | | ✓ | | ✓ | | | ✓ | ✓ | | | | | ✓ | | | | | ✓ | | ✓ | | | | | | ✓ | |
| *Galaxea* | | ✓ | | | | ✓ | | | | | ✓ | | | | | | | | | | ✓ | | | | | | | ✓ | | ✓ | ✓ |
| *Herpolitha* | | | | | ✓ | | | ✓ | | | ✓ | | | | | | | | | | | | | | | | | | | ✓ | |
| *Montipora* | | | ✓ | ✓ | ✓ | | | | | | | | | | | | | | | | | | ✓ | | | | | | | | |
| *Pachyseris* | | | | | ✓ | | | | | | | | | | | | | | | | | | | | | ✓ | | | | | |
| *Pavona* | | | | | | | ✓ | ✓ | | | ✓ | | | | | | | | | | | | | | | | | | | | |
| *Plerogyra* | | | | | | | | ✓ | | | ✓ | | | | | | ✓ | | | | | | | | ✓ | | | | | ✓ | |
| *Pocillopora* | | | | | | | | ✓ | | | ✓ | | | | | ✓ | | | | | | | | | | | | | ✓ | | ✓ |
| *Seriatopora* | | | | | | ✓ | | | ✓ | | ✓ | ✓ | | | | ✓ | | | | | | ✓ | | | ✓ | | | | ✓ | | |
| *Sinularia* | | | | | | | | | | | | | | | | | | | ✓ | ✓ | | | | | | | | | | | |
| *Turbinaria* | | | | | | | | ✓ | | | ✓ | | | | | | ✓ | ✓ | | | | | | | | | | | | | |
| *Xenia* | | ✓ | | | | | | | | | | | | ✓ | ✓ | | | | | | | | | | | | | | | | |

[a]The nominal $m/z$ values listed represent +/− 0.005 mmu. Masses unique to the corresponding genus are shaded.

## EXPERIMENTAL SECTION

**Coral.** In this study, 61 individual coral representing 16 different genera and 22 species were sampled and analyzed. The samples were acquired from CapitalCorals, with identifications made based on morphological features. The species and the number of individuals analyzed were as follows: *Acanthastrea echinata* (2), *Catalaphyllia jardinei* (1), *Duncanopsammia axifuga* (1), *Echinopora lamellosa* (4), *Euphyllia divisa* (1), *Euphyllia paraancora* (2), *Euphyllia paradivisa* (1), *Galaxea fascicularis* (1), *Herpolitha limax* (1), *Montipora confusa* (3), *Monitpora digitata* (5), *Montipora setosa* (3), *Pachyseris rugosa* (1), *Pavona cactus* (3), *Plerogyra sinuosa* (3), *Pocillopora acuta* (10), *Seriatopora hystrix* (8), *Sinularia dura* (4), *Turbinaria peltata* (2), *Turbinaria reniformis* (1), and two species tentatively identified in the poorly defined Xeniidae: *Xenia elongata* (2), and *Xenia umbellata* (2). All specimens were long-term aquarium corals (>2 years) and maintained in a closed aquarium system with artificial seawater (ESV, Hicksville, NY) and artificial LED lighting on a programmed schedule (Reef Breeders, Wakefield, RI). Calcium and alkalinity were maintained with daily supplementation, and corals were fed 3−5 times a week (AminoFeast + amino acid supplement, Coral Feast dry planktonic feed, Willow's Reef, Jacksonville, FL).

**Instrumentation.** Mass spectra for each coral tissue sample were obtained using a DART-SVP ion source (IonSense, Saugus, MA) coupled to a JEOL AccuTOF mass spectrometer (JEOL, Peabody, MA) with a resolving power of 6000 FWHM. The samples were analyzed in positive-ion mode over a mass range $m/z$ 60−1000. The helium gas flow rate of the DART ion source was set to 2.0 L/min, the gas heater temperature was set to 350 °C, and the grid voltage was set to 50 V. The mass spectrometer settings for the ring lens, orifice 1, orifice 2, and peak voltages were 5, 20, and 600 V, respectively.

**Sample Analysis.** Multiple tissue segments of ∼1 mm were taken from each coral and were immediately placed on ice and then stored at −80 °C until analysis. The number of tissue segments/replicates for each individual varied depending on the amount of tissue available. Prior to analysis, the samples were thawed on ice. Each sample was analyzed by suspending and manually rotating it using tweezers in the open-air space between the ion source and mass spectrometer inlet. Along with each replicate, poly(ethylene glycol) 600 (PEG), which served as a mass calibrant, was analyzed, and on average, five replicates were obtained for each sample. The spectra obtained were averaged together to generate a spectrum for each replicate. The mass spectral data were then processed using TSSPro3 software (Schrader Analytical Labs, Detroit, MI). Processing included background subtraction and peak centroiding. The data were then stored in text format.

**Statistical Analysis.** Multivariate statistical analysis was applied to the mass spectral data to assess which algorithm enabled optimal discrimination between the coral genera. All of the genera listed in the Experimental Section were included in a genus-level analysis. Mass Mountaineer software (Mass-spec-software.com, Toronto, Ontario, Canada) was used for spectrum analysis, spectra addition, mass selection, Fisher ratio determination, and Kernel discriminant analysis (KDA)-facilitated classification and discrimination. The relative abundance threshold and mass tolerance were set to 2% and 10 mmu, respectively. To test the accuracy of the model and confirm that this approach can be used for the discrimination of coral genera, leave-one-out cross-validation (LOOCV) was performed.

## RESULTS AND DISCUSSION

The purpose of this study was to explore the hypothesis that genus differentiation of corals could be accomplished by multivariate statistical analysis processing of their chemical fingerprints derived from high-resolution mass spectral analysis using a direct analysis in real time ion source. In the genus differentiation study, both hard and soft coral were analyzed. The soft coral were: *S. dura*, *X. elongata*, and *X. umbellata*. The hard coral were: *A. echinata*, *C. jardinei*, *D. axifuga*, *E. lamellosa*, *E. divisa*, *E. paraancora*, *E. paradivisa*, *G. fascicularis*, *H. limax*, *M. confusa*, *M. digitata*, *M. setosa*, *P. rugosa*, *P. cactus*, *P. sinuosa*, *P. acuta*, *S. hystrix*, *T. peltata*, and *T. reniformis*. The samples used were specimens authenticated by the source (CapitalCorals) using numerous references (Coralsoftheworld.org, JEN Veron, Corals of the World, E. Borneman, Aquatium Corals).[13,58,59] Images of these coral are displayed in Figure 1, Panels A−V.

Figure 2, Panels A−P display DART mass spectra of the analyzed species representative of each of the indicated genera. The mass measurement data corresponding to each spectrum are presented in Supporting Information Tables S-1−S-16.


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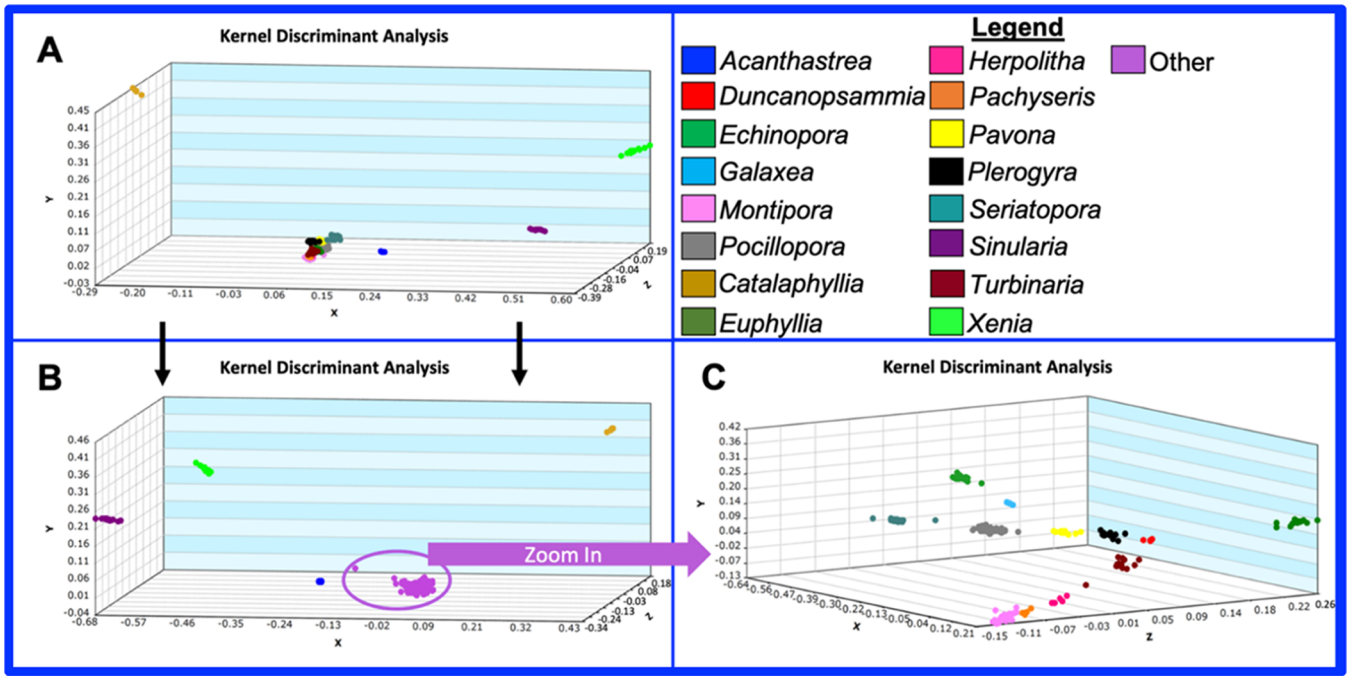


**Figure 3.** Kernel discriminant analysis (KDA) plots derived from the lists of selected discriminating m/z values observed in the DART mass spectra of 16 coral genera presented in SI Tables S-22 and S-23. The various colors correspond to the indicated genera. Panel A: initial genus-level clustering showing all 16 genera (LOOCV: 86.67%); Panel B: plot showing genus-level clustering with the overlapping genera in Panel A grouped together as other (LOOCV: 97.33%); and Panel C: genus-level clustering of only the genera included in other (LOOCV: 93.52%).

Using a 2% relative abundance threshold cutoff, the number of peaks observed in each spectrum varied tremendously depending on the species. *P. cactus* exhibited the least number of peaks (i.e., 14), and *A. echinata* and *S. hystrix* showed the greatest (126). An observed trend among some corals was the similarity in profiles for multiple corals that represented the same species. An example of this is presented in SI Figure S-1, which shows the spectra of four *P. acuta* individuals that were analyzed.

The mass measurement data corresponding to these four spectra are presented in SI Tables S-12 and S-17−S-19. While the relative intensities of the observed $m/z$ values varied, the profiles were remarkably consistent. However, it was also observed in some cases that the mass spectral profiles varied among multiple individuals of the same species. An example of this is presented in SI Figure S-2, which shows the mass spectra from three *P. sinuosa* individuals with the corresponding mass measurement data presented in SI Tables S-11 and S-20−S-21. The results illustrate that although the spectra differ in the number of peaks and some $m/z$ values, they still had in common several $m/z$ values of varying intensities. For example, the individual represented by Figure S-2B has fewer peaks than the others, but almost all of the detected $m/z$ values were also observed in the other individuals represented in Panels A and B. The spectrum in Figure S-2C had a greater number of prominent peaks than the other spectra but included a number of $m/z$ values that were observed in all of the other individuals, including nominal $m/z$ 89, 105, 118, 122, 132, 193, 383, and 518.

The mass spectra of the species representative of each of the genera presented in Figure 2 exhibited a number of major protonated precursor peaks since the mass spectra were acquired under soft ionization conditions. The most prominent $m/z$ values in each spectrum are labeled and displayed in Table 1. A survey of these spectra reveals that some $m/z$ values appeared in multiple genera. Examples include nominal $m/z$ 88 in *Herpolitha, Montipora,* and *Pachyseris*; 89 in *Acanthastrea, Duncanopsammia, Echinopora, Euphyllia, Galaxea,* and *Seriatopora*; 105, which appeared in *Catalaphyllia, Duncanopsammia, Euphyllia, Herpolitha, Pavona, Plerogyra, Pocillopora,* and *Turbinaria*; and 122, which was detected in all of the genera except *Montipora, Pachyseris, Sinularia,* and *Xenia*. *Euphyllia* and *Seriatopra* share $m/z$ 132, and *Duncanopsammia, Echinopora, Euphyllia, Plerogyra,* and *Turbinaria* all have $m/z$ 193. The $m/z$ value 305 is shared in the spectra of *Plerogyra* and *Seriatopora*. *Seriatopora* also shares 381 with *Acanthastrea* and *Pocillopora*. The $m/z$ value 383.3706 ± 0.005 appears in the spectra of *Acanthastrea, Cataphyllia, Duncanopsammia, Echinopora, Euphyllia, Galaxea, Herpolitha, Pachyseris,* and *Plerogyra*. Overall, many of the metabolites represented by the observed $m/z$ values were common to several species, although they varied in intensity between genera. However, in general, it was the $m/z$ values at the lower intensities that introduced the variation that enabled distinctions to be made between genera and resulted in each genus exhibiting a unique chemical fingerprint. The identities of the compounds represented by these masses remain unknown and are the subject of ongoing studies.

Of the species analyzed, several examples were members of the same genus, and this provided the opportunity to assess the similarities between them. These included *E. divisa, E. paraancora,* and *E. paradivisa* of the *Euphyllia* genus; *M. confusa, M. digitata,* and *M. setosa* of the *Montipora* genus; *T. peltata* and *T. reniformis* of the *Turbinaria* genus, and *X. elongata* and *X. umbellata* of the *Xenia* genus. A consistent trend in each of these cases was the appearance of several shared $m/z$ values. The *Euphyllia* species shared almost all of their prominent $m/z$ values, including nominal $m/z$ 303, which


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Anal. Chem. 2021, 93, 15306−15314

was unique to the genus (i.e., it did not appear in the spectra of any of the other genera analyzed). Interestingly, other $m/z$ values were observed that were unique to each of the individual species represented. These included nominal $m/z$ 73 in *E. divisa*, $m/z$ 273 in *E. paraancora*, and $m/z$ values 135 and 317 in *E. paradivisa*. The *Montipora* species shared a number of prominent $m/z$ values, including the genus unique $m/z$ 74, 79, and 298. Similar to what was observed in *Euphyllia*, there were $m/z$ values that were unique to species within the *Montipora* genus. For example, *M. confusa* had the species-unique $m/z$ values 136 and 518; only *M. digitata* had $m/z$ 284.2388; and $m/z$ 253.1818 only appeared in *M. setosa*. The *Turbinaria* species followed the same trend: there were shared prominent $m/z$ values among the represented species, and also other valuess that were species-specific. The latter category included $m/z$ 199 in *T. peltata*, which did not appear in *T. reniformis*. *T. reniformis* had $m/z$ values 135, 273, and 381.3544 that did not appear in *T. peltata*. The last of the genera analyzed for which multiple species were represented was *Xenia*. Prominent shared $m/z$ values included nominal 149, while species-specific attributes included 123.0542, 125.0629, 315.2004, 327.0146, and 345 for *X. elongata*; and 166 for *X. umbellata*. Overall, the observation of shared prominent $m/z$ values among members of multiple species within a genus, and which differ between genera, suggests that they can be distinguished from one another at the genus level based on their mass spectral profiles. The unique $m/z$ values between species of a genus also indicate the possibility that the presence of these compounds may enable classification of species within a genus based on DART-HRMS-facilitated chemical profiling.

While the visually apparent inter-genus differences observed in the mass spectra indicated genus-specific chemical profiles that might be used for genus identification, a less subjective approach to genus attribution was explored. Mass spectra representing all 16 genera (300 in total) were subjected to multivariate statistical analysis. First, the Fisher ratio algorithm was applied to reveal the masses that were most important for discrimination between genera, and the discriminating masses were used to perform Kernel discriminant analysis (KDA). The masses are shown in Supporting Information Table S-22, and the resulting KDA plot is shown in Figure 3. The plot in Panel A readily illustrates the separation and distinct clustering of the *Acanthastrea*, *Catalaphyllia*, *Sinularia*, and *Xenia* genera. The remaining twelve genera, including *Duncanopsammia*, *Echinopora*, *Euphyllia*, *Galaxea*, *Herpolitha*, *Montipora*, *Pachyseris*, *Pavona*, *Plerogyra*, *Pocillopora*, *Seriatopora*, and *Turbinaria*, also exhibited clustering, although they were much closer together and appeared overlapped in the plot. The leave-one-out cross-validation (LOOCV) for this plot revealed a classification accuracy for the model of 86.67%. The overlapping genera in Panel A (grouped together and referred to as "other") were subjected to another iteration of KDA using the same discriminating masses listed in SI Table S-22. This furnished the plot shown in Figure 3 Panel B. The LOOCV for this plot showed a classification accuracy of 97.33%, which indicates that there is a good discrimination between the four genera that were separated and the rest of the genera. Lastly, a third iteration of KDA was performed only on the genera that were grouped as "other" in the plot shown in Panel B. This can be viewed as a magnification of the overlapping genera. For this analysis, the Fisher ratio algorithm was applied to only the genera included in the "other" category, and this yielded the discriminating masses listed in SI Table S-23. The resulting KDA plot is presented in Figure 3 Panel C. It illustrates the separation and distinct clustering of the genera, and it exhibited a LOOCV of 93.52%. This indicates proof of concept that molecular fingerprinting can be successfully used to provide genus-level information on coral tissue. Of note is the fact that this approach to genus attribution from tissue sample analysis does not require *a priori* knowledge of the molecular identities of the $m/z$ values that enable the genera to be separated from one another. Information about the structures of these compounds could not be determined because of the small coral sample sizes available (<mg), which precluded their isolation and characterization. Nevertheless, their presence hints at the possibility of genus-specific biomarkers whose detection could serve as a means of identification and which can be further investigated through the analysis of larger amounts of bulk tissue. For example, the *Acanthastrea* species spectra had unique and prominent $m/z$ values at 60.0484 and 119.0830 that were not detected in the other genera. Additional similar examples of $m/z$ values unique to various genera include nominal: $m/z$ 145 (*Catalaphyllia*); $m/z$ 319 (*Echinopora*); $m/z$ 303 (*Euphyllia*); $m/z$ values 261 and 336 (*Galaxea*); $m/z$ values 74, 79, and 298 (*Montipora*); $m/z$ 313 (*Pachyseris*); $m/z$ 100 (*Pavona*); $m/z$ 118 (*Seriatopora*); $m/z$ values 205 and 217 (*Sinularia*); $m/z$ 199 (*Turbinaria*); and $m/z$ values 149 and 166 (*Xenia*).

## CONCLUSIONS

Analysis by DART-HRMS of the tissue of 61 coral samples representative of 22 coral species falling within 16 genera showed proof of concept for this technique to be a promising method for rapid sampling that furnishes genus-specific chemical fingerprints that can be used to infer genus identity. Samples comprised of ∼1 mg of tissue could be analyzed by DART-HRMS in their native form without any preprocessing, and each analysis was accomplished in ∼5 s. The observed small-molecule profiles exhibited visually apparent inter-genus differences. Furthermore, in several cases where multiple species within a single genus were analyzed, including *Euphyllia*, *Montipora*, *Turbinaria*, and *Xenia*, chemical profile consistencies between them were observed, which distinguished them from the species of the other genera. Determination of Fisher ratios revealed a subset of $m/z$ values, which, when subjected to kernel discriminate analysis, resulted in a prediction model that enabled genus-level differentiation with an accuracy varying between 86.67 and 97.33%, depending on the species. The results pave the way for the creation of an expanding database of genus-level chemical profiles that could be used to rapidly and accurately differentiate coral genera. As these chemical profiles are correlated to the expanding list of coral genomes, and the relationship between chemical phenotype and morphological plasticity is mapped, the approach described herein would find increasing utility and potentially be extended to the accomplishment of coral species identification.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c02519.

Additional mass spectra of multiple individuals analyzed from the coral species *Pocillopra acuta* and *Plerogyra sinuosa* and tables containing information on the coral



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Anal. Chem. 2021, 93, 15306−15314

spectra, including accurate masses and relative intensities of the peaks, and the feature masses for KDA differentiation and classification (PDF)

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### Notes

The authors declare no competing financial interest.

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